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Review of a Safety Assessment in Mice Administered a Dietary Supplement Containing Aegeline (OxyELITE Pro New Formula)

Cyril Willson*

EuSci LLC, Nebraska, USA

**Corresponding e-mail: cmwillson@gmail.com*

ABSTRACT

The use of animal models in scientific research is considered by many to be an absolute necessity, while some have questioned whether there are better alternatives. Nonetheless, animal models are currently a foundational piece of modern research practices. However, amongst the criticisms leveled at supporters of animal research, those which even supporters concede are necessary include sound methodology and design of such studies along with transparency for results. In Part 3 of a 3 Part series, a recently published safety assessment in mice administered a recreated OxyELITE Pro formulation is reviewed. Unfortunately, this study serves as yet another example where animal research has fallen short, suffering from numerous design and methodological issues and a lack of transparency in reporting results.

Keywords: Body surface area scaling, Interspecies scaling, Pharmacokinetics

INTRODUCTION

The use of animal models as a predictive means of assessing the potential toxicological and pharmacological effects of chemicals in humans is a cornerstone of modern research [1]. However, more recently, some have questioned the benefits of using animal models for such purposes, noting the discordant results between animals and humans whether toxicologically or pharmacologically [1-4]. While moving toward a less costly, more humane and predictively superior model or models is ideal, animal models are the current standard despite limitations. Some authors have noted that a partial solution (i.e., solutions outside of inter-species differences that will inherently result in divergent results) to the controversy regarding the applicability of animal models to humans can ultimately be found by addressing methodological and design errors in such studies along with transparency and accountability for data [1].

OxyELITE Pro or rather, a reconstructed version of one of the formulas containing aegeline, yohimbine, higenamine, and caffeine was recently studied by Miousse, et al., in mice [5]. A review of the published data reveals numerous shortcomings that are addressed herein which provide an example of a case where design and methodological errors led to inappropriate dosing of the compounds administered along with interpretational errors by the authors.

Literature Search

As part of a general (non-systematic) review, PubMed and Google Scholar were searched for original research articles, reviews, reports and letters to the editor which included an analysis of aegeline or OxyELITE Pro, and hepatotoxicity or liver injury. Search terms included: OxyELITE Pro, OEP, aegeline, hepatotoxicity, liver, liver injury. Publications retrieved were analyzed with respect to any association between aegeline or OxyELITE Pro and hepatotoxicity.

A Safety Assessment of OxyELITE Pro New Formula in Mice

Misleading claims: Miousse, et al., report the results of a "safety assessment" study where one of the three "new formulas" (i.e., referring to those which did not contain 1,3-dimethylamylamine (DMAA), but instead, aegeline) of OxyELITE Pro, also referred to as OxyELITE Pro New Formula or OEP-NF containing aegeline, caffeine, yohimbine, and higenamine, was reconstructed and examined in CD-1 and B6C3F1 mice for potential hepatotoxicity [5]. The authors hypothesize that while these individual ingredients alone do not appear to be hepatotoxic, their combination could lead to adverse findings. First, the authors make erroneous and misleading statements; for example, the authors state that Arseculeratne, et al., utilized mice, when in fact they were rats [6]. The authors also, like Klontz, et al.,

neglect to include data showing that other than the questionable results of Arseculeratne, et al., *A. marmelos* and aegeline have not otherwise shown hepatotoxic effects and in fact have been shown to be hepatoprotective (Tables 1 and 2 of Part 1) [7,8]. The authors also discuss a study by Mohammed, et al., stating that the authors found naturally derived aegeline to be cytotoxic in HepG2 cells in a study evaluating the anti-cancer potential of various compounds from *A. marmelos* against several cancer cells lines [9]. The authors cite this study as apparent evidence that aegeline may be hepatotoxic. However, this is misleading as the authors performed an MTT assay, which as Galluzzi, et al., and others have pointed out is an indirect and potentially unreliable method for measuring cell death which could lead to erroneous results, as cells are not actually counted [10,11]. Nonetheless, the concentration used (i.e., 336.3 µmol), assuming linear kinetics, would require a 3,000 mg/kg dose (i.e., 125 times the amount in a 1x dose of OEP-NF used by Miousse, et al., [5]) of aegeline in mice to reach a similar concentration and is unlikely to be relevant to *in vivo* administration [12]. Furthermore, chlorogenic acid, a commonly consumed polyphenol ubiquitously found in the human diet in fruit, vegetables, coffee, and tea was shown to cause cell death in HepG2 cells, as were strawberry and plum extracts as well as curcumin [13,14]. Last, it is important to note that compounds may demonstrate cytotoxicity to cancerous cell lines such as HepG2 while having no effect on normal cells lines [14-16]. This is, in fact, is a desired characteristic of a potential anti-cancer compound.

The authors reference Waluga, et al., claiming that it shows the ability of caffeine to intensify the hemodynamic effects of yohimbine [17]. However, the study administered a placebo; caffeine and ephedrine; and caffeine, ephedrine, and yohimbine. Thus, changes seen in the third group, based upon the study design, do not allow one to draw such a conclusion that Miousse, et al., have done [5]. In fact, Waluga, et al., make no such statements and simply caution against the combination of the three ingredients [17]. It is just as possible that there was a negative interaction between ephedrine and yohimbine, considering norepinephrine's inhibitory feedback upon its own release [18], via activation of alpha-2 adrenergic receptors; something which would be overridden if ephedrine is consumed with yohimbine. In any event, unless the study was designed with positive control groups receiving each individual ingredient alone and in combination, there is no way to know for certain. The claim that yohimbine and caffeine are known to have such a negative interaction, however, is unfounded. Furthermore, the relevance of interactions and their effects on hemodynamic variables and cardiovascular function to effects upon the liver is questionable. Finally, others have evaluated the hemodynamic effects of a supplement which contained caffeine, higenamine, and yohimbine, as compared to caffeine alone, placebo, or higenamine. There was no significant difference in heart rate or blood pressure in those consuming the combination of caffeine, higenamine, and yohimbine as compared to placebo or caffeine alone, which further demonstrates the lack of any negative interaction between the ingredients [19].

Inappropriate Dose Scaling and Neglect of Pharmacokinetic/Toxicokinetic Differences between Humans and Mice: Miousse, et al., attempt the calculation of a "mouse equivalent dose" or MED, derived from the dose humans received after ingesting the maximum daily dose of 3 capsules of an OEP-NF formula [5]. Miousse, et al., perform a dose extrapolation, which Blanchard and Smoliga have discredited, pointing out that it is erroneous and ill-supported, mainly due to a misinterpretation of Food and Drug Administration (FDA) guidelines for first in human dosing [20]. At best, such a method is considered crude and preliminary as it does not account for differences in pharmacokinetics or pharmacodynamics between species [20-23]. The authors first use the conversion inappropriately but then further confound matters by using the formula in reverse. For example, with respect to the ingredient caffeine, which was included in OEP-NF at 400 mg per 3 capsules, the dose ingested by a 70 kg human is approximately 5.7 mg/kg. The authors take this figure and simply multiply it by a factor of 12.3 to give what they claim is the MED, which becomes the 1x group and consequently, the 1.5x, 2x, 3x, and 10x groups received approximately 105, 140, 210 and 700 mg/kg of caffeine, respectively. The 1x dose for mice would be around 2.3 mg/kg for yohimbine, while aegeline's would be approximately 24 mg/kg, as examples. Notably, despite performing a toxicologically based study, the authors neglect to use a default factor most commonly used to determine a "toxicologically equivalent" dose, which involves scaling of doses by 3/4-power of bodyweight or BW0.75 [23,24]. Despite the fact that this method also does not consider toxicokinetic and toxicodynamic differences between species for a given chemical, it is at least consistent with mainstream toxicology. Notably, the scaling factor is substantially lower than that used by the authors. For example, in mice, a dose of caffeine considered, "toxicologically equivalent" to 5.7 mg/kg in humans would equate to approximately 40 mg/kg [23,24], which is over 40% lower than that used by Miousse, et al., [5]. Instead, the authors have used the FDA guidance document, whose singular purpose is to obtain a human equivalent dose (HED) derived from a no observed adverse effect level (NOAEL) in an appropriate animal model by scaling with body surface area

(BSA) [20]. The entire purpose of this conversion is to provide a starting point for a (hopefully) safe dose in humans when a compound is being given to humans for the first time, although it has even failed for this purpose at times [20]. Even ignoring the mistake of using the equation in reverse, it would not be a NOAEL that is being determined from an HED but instead, as the FDA points out, the pharmacologically active dose (PAD), which is the lowest dose tested in an animal species with the intended pharmacologic activity/therapeutic effect. The NOAEL and PAD are entirely different, yet the authors apparently confuse the two or consider them interchangeable, which they are not [20]. Even ignoring the total formula of OEP-NF, it is well established that caffeine alone, at a dose of 400 mg for a 70 kg adult is a pharmacologically active dose (PAD), as opposed to a NOAEL [25]. Yet, even the amount of caffeine in a single capsule of OEP-NF (i.e., 135 mg) is known to be pharmacologically active [25]. Furthermore, as little as 2 capsules in humans given an OxyELITE Pro-like formula with yohimbine, caffeine, and higenamine stimulated lipolysis and energy expenditure, thus this would also qualify as the PAD [26]. The label directions for OEP-NF inform the consumer that they may use between 1-3 capsules daily. Yet, the authors chose to use 3 capsules instead of 1 or 2 capsules, despite the fact that these doses are also pharmacologically active. Thus, not only did the authors fail to use the PAD instead of a NOAEL, they failed to use the minimum dose known to have pharmacological effects and instead, simply used the maximum number of capsules allowed for consumption according to the label. Just as concerning is the fact that while a total daily intake of 400 mg of caffeine is considered unlikely to cause serious harm in adult humans, the authors took no steps to confirm that the doses employed via their conversion factor were similarly unlikely to cause serious harm in mice. Of course, this also exempliies the fact that the authors were also incorrectly performing dose scaling for pharmacological purposes, rather than toxicological purposes, a notable error.

In performing such a calculation, the authors ignore not only the warnings of Blanchard and Smoliga but those of Sharma and McNeill who point out major issues (i.e., lack of consideration for pharmacokinetics/pharmacodynamics, use of a NOAEL instead of PAD and an increased estimated dose) when it comes to extrapolation from a human to an animal using the same conversion factors [20,21].

Finally, even in the paper cited by the authors as justification for this calculation, Wojcikowski and Gobe also make the same warning about ignoring the potential pharmacokinetic and pharmacodynamic differences between species, a warning echoed by toxicologists as well, stating: “There are numerous arguments against the use of animal studies on medicinal herbs or drugs, many of which involve the fact that animal studies do not necessarily predict what will happen in humans. It is true that a significant interspecies variation in any of the components of pharmacokinetics and pharmacodynamics may result in the inappropriate extrapolation of animal dose to humans” [22,23].

It is evident that the authors did not consider pharmacokinetic/toxicokinetic or pharmacodynamic/toxicodynamic differences between humans and the mice used in the study when extrapolating using BSA. For example, the peak plasma concentration of caffeine in humans varies but has been shown to fall around 9 to 10 mg/L after a 350 to 400 mg dose (i.e., 400 mg or 5.7 mg/kg dose in 3 capsules of OEP-NF for a 70 kg human) [27-30]. These data are in agreement with the summary by Arnaud in 2011, which indicates the mean peak plasma concentration in humans after oral or intravenous administration of doses between 5-8 mg/kg is between 8-10 mg/L [31]. In mice, however, a 20 mg/kg dose given orally produces a peak plasma concentration of around 14.5 mg/L [32]. This is in good agreement with the data by Sakai, et al., who found a peak plasma concentration of approximately 1.07 mg/L after a 1.6 mg/kg oral dose in mice; assuming linear kinetics up to 100 mg/kg (i.e., caffeine is known to display linear kinetics up to 100 mg/kg in mice [31], but it is unknown if this remains at doses above this level, which incidentally, is yet another issue with the caffeine doses above 100 mg/kg employed by Miousse, et al., [5]), a dose of 16 mg/kg would have been expected to yield a peak plasma concentration of around 10.7 mg/L [31-33]. Clearly, even a 20 mg/kg dose in mice would have more than equaled the same peak plasma concentrations and internal exposure in a human consuming 400 mg in OEP (i.e., 5.7 mg/kg), yet, because Miousse, et al., used a calculation for a purpose which it was not intended, the mice instead received approximately 70 mg/kg [5]. That is at least 3.5 times greater than what was needed to achieve a similar internal exposure to caffeine in mice as that in humans and more than 40% higher than a standard default factor for toxicological studies assuming no significant toxicokinetic/toxicodynamic differences between species [23]. Blanchard and Smoliga, point out a similar example using the dietary supplement resveratrol, showing that using the conversion factor that Miousse, et al., use, results in a grossly overestimated dose in mice, validating the warnings also expressed by Sharma and McNeil, regarding an increased estimated dose using the conversion employed by Miousse, et al., [5,20,21].

Toxic doses of caffeine administered to mice: The most obvious indication that the scaling performed by Miousse, et al., was inappropriate is the fact that the dose of caffeine given to the mice in the 2x and 3x groups, was close to or beyond the acute LD50 value [5,34]. For example, the acute LD50 value for the CD-1 mice used in this study is between 127-137 mg/kg, thus doses of 140 and 210 mg/kg in the 2x and 3x groups would also be expected to cause toxicity based upon the caffeine content alone [34], while the 10x groups would be expected to most likely result in 100% lethality as it is more than 5 times greater than the acute LD50 [34]. Others have warned that an acutely toxic dose should not be used for sub-chronic toxicity studies [23]. The authors in apparent acknowledgment of this issue, seem to reason that although some groups of mice clearly received lethal doses of caffeine, those receiving doses just below the LD50 (e.g. 1x and 1.5x groups) would not have been influenced [5]. Of course, the authors neglect to consider that the LD50 is merely a measure of acute toxicity (i.e., in the form of lethality) and does not represent the absolute point of lethality; rather it represents the dose needed to cause death in 50% of animals after a single dose [35]. The LD10 (i.e., the dose needed to cause death in 10% of animals), while unknown, would most certainly be even lower than these figures and the toxic dose 50 or TD50 (i.e., the dose which produces toxicity in 50% of a group) even lower still, considering a typical monotonic dose-response curve [35]. After all, the LD50 is simply a measure of acute toxicity after a single dose in the form of mortality/lethality, rather than a measure of toxicity after repeated dosing (i.e., sub-acute or sub-chronic toxicity as in these studies). Furthermore, and perhaps, more importantly, the authors ignore the fact that the LD50 is a measure of mortality/lethality, not morbidity (i.e., toxic or adverse effects occurring below the threshold for lethality) because even if death does not occur, the animals can still suffer toxicity short of death [35,36]. Walum points this out, stating that, "*the value refers only to mortality and is illustrative of no other clinical expression of toxicity*" [36]. Although, Zbinden and Flury-Roversi have pointed this out decades ago [37]. Zbinden and Flury-Roversi also point to the flaw of assuming that a dose which is simply below the acute LD50 will be tolerated with repeated dosing, using an example of the drug dexamethasone, where it was shown that rodents were not able to tolerate doses even 1,700 times lower than the acute LD50, when given repeatedly [37].

An example of dose scaling in rats: To further illustrate the flaws of the dose extrapolation performed by the authors, one can also reference studies in rats. Using the same conversion formula and arguments applied by the authors, rats could simply be given (i.e., 5.7 mg/kg x 6.2) approximately 35 mg/kg of caffeine to equal a 1x dose, 105 mg/kg for a 3x dose and 350 mg for a 10x dose [5]. However, this is clearly an excessive and erroneous conversion when compared with the data published by Latini, et al., which found the average peak plasma concentration after oral administration of 10 mg/kg of caffeine in rats to be approximately 12.2 μg/ml, which is approximately the same as what a human experiences after a 5.6 mg/kg ingestion of caffeine [30,38]. Yet, if using the calculation employed by Miousse, et al., the dose would have been 3.5 times greater [5]. These data again show the inaccurate dose conversion by relying upon BSA when there are clear pharmacokinetic differences between rodents and humans regarding caffeine; reversing the formula, and confusing a PAD for a NOAEL to come up with a supposed MED. Arnaud has even warned about the practice of dose extrapolation with caffeine noting that, "*Important pharmacokinetic differences have been reported between animal species, making the extrapolation between species difficult*" [31].

An example of caffeine toxicity in rats using inappropriate dose scaling: Returning back to the example in rats, an LD50 value of 192 mg/kg has been determined previously, thus, if the authors applied a 10-fold increase to their claimed equivalent dose, it would equal a dose of 350 mg/kg, which they claim should be a dose which does not result in any adverse effects in the animal [39]. However, this dose is lethal to rats [39]. As noted by Boyd after rats were given lethal doses of caffeine, they displayed symptoms such as swollen and inflamed eyelids, schizophreniform withdrawal, hyperreflexia, vertigo, ataxia, loss of body weight, gastroenteritis, hepatitis, nephritis, toxic effects upon the heart, spleen, pancreas, thymus gland and adrenal glands, while they ultimately succumbed to respiratory failure or cardiovascular collapse [39]. However, even these effects are only from the acute administration. As discussed, the LD50 value is merely a measure of acute toxicity (mortality/lethality) and in fact, the LD50 value may be much lower if an animal is subjected to repeated administration. Indeed, this is what Boyd et al. found in another study [40]. The repeat-dose LD50 in rats given caffeine over a 100 day period demonstrated an LD50 value of 150 mg/kg, significantly lower than the acute single dose LD50 of 192 mg/kg [39,40]. Similarly, these rats displayed symptoms such as toxic nephritis, hepatitis, myositis, thyroiditis, and loss of red pulp in the spleen [40]. In those receiving as little as 158-181 mg/kg, histopathological studies in the liver demonstrated changes such as sinusoidal congestion, cloudy swelling and congestion, while clear histopathological changes were also noted in the brain, skeletal muscle, heart, kidneys, GI tract, lung, adrenal gland, spleen, thymus and thyroid glands [40]. Interestingly, in a clear example of why the LD50

is only an indication of mortality/lethality, not morbidity or sub-lethal toxicity, rats given an average dose on the order of approximately 110 mg/kg day, which was the highest dose (i.e., a sub-lethal dose) that did not produce any deaths over 100 days, still produced significant toxicity/adverse effects [35-37,40]. These rats experienced cerebral hyperemia, occasional psychotic-like self-mutilation (i.e., biting and mutilating its own feet or tail), gastric ulcers, and hypertrophy or enlargement of the salivary glands, GI tract, liver, heart, kidneys, and lungs, despite having in general, a normal appearance [40]. This is a clear example of the authors' error. For example, if rats in a study were given 3x what Miousse, et al., claim is the equivalent dose to humans, that would be approximately 105 mg/kg, which would have resulted in multi-organ toxicity and could have led to elevations of ALT and AST, which could have come from the liver or more likely, other non-hepatic sources such as the brain, kidneys, skeletal muscle or heart as ALT and AST are not specific only to the liver but may also rise in response to other tissue damage [5,40-42]. Indeed, demonstrating the errors of Miousse, et al., a recent study in albino rats administered 50, 100, 190 and 300 mg/kg caffeine for 28 days and found that all doses, including only 50 mg/kg (i.e., slightly less than 1.5x what Miousse, et al., claim is a dose equivalent to 5.7 mg/kg in humans), produced a statistically significant but moderate increase in ALT and AST, yet no increase in ALP or bilirubin, which actually decreased, indicating the elevation may have come from tissues other than the liver [43]. The authors also noted indications of kidney damage and a reduction in weight gain relative to control, also noting that the reduction in weight gain in rodents by caffeine is well-described by others [43]. Interestingly, in rabbits, a dose of 80 mg/kg caused increases in ALT and alkaline phosphatase in a 28-day study, despite the lack of any histopathological changes to the liver [44].

Inappropriate interpretation and application of safety factors: Miousse, et al., comment regarding their surprise that only 3 and 10 times what they claim is the recommended dose equivalent would produce harm, arguing that these doses are well within a 100-fold uncertainty factor recommended when designing herbal dietary supplement safety studies [5]. The authors cite the work of Schilter, et al., as apparent support for this claim, but Schilter, et al., make no mention of the approach used by Miousse et al., [5,45]. Instead, Schilter, et al., detail a commonly accepted method for the determination of a potentially safe dose for a given substance to be consumed by humans (e.g., the acceptable daily intake or ADI) by determining a NOAEL in animal models, which is subsequently divided by a 100-fold uncertainty factor to account for inter and intra-species differences in toxicological response [45]. Here again, it is apparent that Miousse, et al., believe that one may simply substitute a NOAEL in animals for the PAD in humans, that is, the dose consumed by humans for a given product could simply be converted to an equivalent dose in an animal model and animals should be free from adverse effects at a dose up to 100-fold greater. In effect, Miousse, et al., erroneously believe that for example, an intake of 5.7 mg/kg of caffeine in humans represents a NOAEL and thus it can be converted to an animal equivalent dose by using a BSA scaling formula in reverse, yielding an animal equivalent dose from that value, which the authors believe is 70 mg/kg caffeine for mice. Subsequently, the authors erroneously believe that since this dose is a NOAEL, animals should be able to consume 100-fold greater quantities without harm. Such arguments are unprecedented and are readily demonstrated as faulty. For example, as already noted 10x a 70 mg/kg dose (i.e., 700 mg/kg) of caffeine is obviously lethal to mice [34]; 100x a 70 mg/kg dose (i.e., 7,000 mg/kg) of caffeine is also obviously lethal to mice. Even assuming a 5.7 mg/kg dose in humans as a NOAEL a 100-fold increase (i.e., 570 mg/kg) is clearly lethal to mice [34]. A 5.7 mg/kg ADI for humans derived from a 100-fold safety factor would also require a NOAEL of 570 mg/kg in mice, which is again, lethal [34]. Such arguments also demonstrate a fundamental misunderstanding of basic toxicological principles. For example, even if the authors had correctly determined and appropriately scaled a NOAEL rather than a PAD, such doses (e.g., 3x and 10x) beyond the NOAEL are by definition, capable of causing observable adverse effects [35]. The authors also fail to recognize that safety factors are inherently scaled downward, not upward as their purpose is to determine a safe dose.

Caffeine toxicity in humans: As it has been demonstrated previously, the doses that Miousse, et al., administered were most certainly not equivalent to what humans ingest, resulting in lethal and toxic doses of caffeine being given, including a lethal dose in the 3x dose group of mice [5,34]. Yet a claimed human equivalent dose according to Miousse, et al., a 1,200 mg (i.e., 3x 400 mg) or 17 mg/kg dose (i.e., for a 70 kg adult) is well below the known lethal dose for normal adult humans [5,46-51]. In addition, the notion that a 10-fold overdose beyond a PAD or even a NOAEL would cause harm should not be surprising, especially with caffeine, considering the somewhat narrow margin between well-tolerated doses and those producing toxicity. Even if the authors had more appropriately dosed the mice with 20 mg/kg of caffeine, a 10-fold increase to 200 mg/kg would still have caused lethality in some animals [34]. A 200-400 mg dose of caffeine or 2-4 cups of coffee is associated with positive benefits or a lack of risk for

serious harm in normal healthy adults, yet a 10-fold increase (i.e., 20-40 cups) to 2,000 to 4,000 mg, would place many individuals in the hospital for supportive treatment [46-51]. A 1,000 mg dose of acetaminophen may provide pain relief but a 10,000 mg dose could cause severe liver injury [52]. As noted previously, even a 3 or 10-fold dose greater than the NOAEL would by definition, be expected to cause adverse effects. Even essential molecules may cause toxicity in such instances. While most humans consume less than 2 L of liquid or water daily, a 10-fold increase would likely be lethal [53-55]. This is not an anomaly, it is a tenet given to us by the father of toxicology, Philippus Theophrastus Aureolus Bombastus von Hohenheim, which most know as, "Paracelsus", who stated, "*What is there that is not poison? All things are poison and nothing (is) without poison. Solely the dose determines that a thing is not a poison*" [56].

Inappropriate dose scaling of yohimbine: Unfortunately, similar to caffeine, there appears to be a lack of consideration for the pharmacokinetics/toxicokinetics of yohimbine in rodents, versus humans. For example, in humans, the elimination half-life of yohimbine is generally around 1 hour, while in mice and rats, it is considerably longer at approximately 6 hours and over 16 hours, respectively [57-59]. Some authors have commented that such differences underscore the fact that it is difficult to compare data from two species [60]. In any event, this lack of consideration for the pharmacokinetics of yohimbine and the incorrect use of BSA for a conversion factor could also have led to toxic concentrations of the compound either on its own or after potentially accumulating [23]. While the oral LD50 for yohimbine is approximately 43 mg/kg in mice, as discussed previously, this is simply an acute, single-dose assessment of lethality [61]. It does not represent a repeated dose LD50 nor does it indicate that lethality or sub-lethal toxicity (i.e., adverse effects) do not occur at lower doses [35-37].

Inappropriate study design using insufficient blood chemistry: While major issues have already been discussed with the design of the study, there are additional issues. For example, the authors in Miousse, et al., failed to follow proper guidelines for blood chemistry tests in animal toxicity studies, which could have helped distinguish whether any modest increases in ALT/AST were coming from the liver or non-hepatic tissues [62-65]. Furthermore, as previously noted, the rather broad organ toxicity seen when rodents overdose with caffeine can also give rise to elevations of ALT/AST as those enzymes are found not just in the liver, but the brain, lung, pancreas, kidney, heart and skeletal muscle [41-43]. It does not appear the authors followed proper and basic protocols as determined by guidelines issued by respected organizations, which indicate that for animal studies such as these, one should measure two hepatocellular and two hepatobiliary markers or at least 3 enzymes indicative of hepatocellular effects (i.e., the authors measured only two enzymes), in addition to other clinical chemistry such as blood urea nitrogen, creatinine and albumin, which for example, allows for a better determination as to whether ALT and AST are rising from extra-hepatic (e.g., brain, skeletal muscle, heart, kidney, or elsewhere) sources as this is a major limitation of relying only upon ALT and AST as they can arise from other tissues [41,42,62-65]. These and other basic blood chemistry tests (e.g., bilirubin) were inexplicably ignored by this group. The European Medicine Agency specifically states that bilirubin is needed along with ALT and AST, the reason being that while it is considered a hepatobiliary marker, it allows for the differentiation between ALT/AST rising from non-hepatic and hepatic tissues; this trio, is said to be "*the most relevant signal of liver toxicity*" [62].

The fact that the authors chose ALT and AST while neglecting to use any other well-established indicators such as alkaline phosphatase or bilirubin is particularly problematic. While the group does evaluate microRNA 122 or miR-122 and the liver/body weight ratios, these too are problematic. For example, while miR-122 was once thought to be an emerging contender for a new biomarker for liver injury, recent evidence indicates that it can also be actively released or regulated and does not appear to merely reflect passive release due to hepatocyte damage [66-68]. While miR-122 is found primarily in the liver, it is known to regulate metabolic pathways including cholesterol biosynthesis and fatty acid synthesis as well as oxidation [66,69-71]. Furthermore, some have indicated that free fatty acids may regulate miR-122 expression, which in turn causes a shift which favors β-oxidation by inhibiting enzymes necessary for triglyceride synthesis [66]. This would explain why miR-122 could have risen in some animals being fed a supplement designed to stimulate lipolysis which in turn increases free fatty acid levels [26], especially considering the fact that Miousse, et al., fail to present any direct evidence that absolute liver weights and histopathology reflected toxicity [72]. In fact, it has more recently been shown that free fatty acids increased expression of miR-122 in the liver of mice and induced secretion of miR-122 from the liver to blood where it entered muscle and adipose tissue and reduced expression of genes involved in triglyceride synthesis, providing strong evidence for such a role for miR-

122 [68]. Furthermore, this group found that administration of a beta 3-adrenergic agonist which stimulates lipolysis also increased miR-122 and finally, they also found a positive correlation between free fatty acids and miR-122 in the plasma of healthy humans after fasting [68]. These effects occurred without any occurrence of hepatocyte death, demonstrating the increased miR-122 was due to active or regulated release rather than passive release [68]. These and other data have caused Chai, et al., to conclude that miR-122 can act as a hormone or signaling molecule which is actively released or regulated and represents a new molecular target for reducing triglyceride levels in those with metabolic syndrome [68]. Interestingly, coffee polyphenols were shown to reduce body fat accumulation in mice, while also showing an increase in miR-122 levels in liver [73]. Fatty acids have also been shown to upregulate miR-122 expression in human embryonic stem cells, while a high-fat diet designed to induce non-alcoholic fatty liver disease (NAFLD) caused a down-regulation of miR-122 in rat liver [74].

It is interesting to note that Chai, et al., found increased miR-122 after administration of a beta 3-adrenergic agonist, as aegeline, one of the ingredients in OEP-NF has been proposed as a beta 3-adrenergic agonist with confirmed activity, while higenamine, another ingredient, also has beta-adrenergic activity [26,68,75,76]. Of course, two of the other ingredients in OEP-NF, caffeine, and yohimbine are also known to stimulate lipolysis [77-79]. Others have also noted that potential biomarkers such as miR-122 have been more thoroughly assessed after intrinsic hepatotoxic agents such as acetaminophen, as opposed to those known to cause idiosyncratic DILI, while also noting that the best potential role is complementary, rather than as a replacement for established clinical tests [80]. On the other hand, like so many variables, there was no consistent rise in the mice of Miousse, et al., study (i.e., only the male CD-1, 4-week mice with the highest dose and the female B6C3F1, 13-week mice but only in those receiving the lowest or 1x dose) and the miR-122 didn't corroborate a change in ALT/AST nor apparent histopathology (i.e., unusually, the authors do not report statistical comparisons nor actual histopathological data for the groups of mice leaving the reader to presume that if there were a change, it would have been directly reported) [72,81]. Furthermore, the 1x and highest dose groups often displayed a decrease in miR-122 relative to control. In any event, the discoveries (i.e., it can be actively released or regulated) regarding miR-122 along with the degree of inter and intra-variability has led the predictive safety testing consortium (PSTC) to deprioritize miR-122 as a DILI biomarker [67].

Misleading use of the liver to body weight ratio as an indicator of liver hypertrophy: In addition to other issues, the authors' use of the liver to body weight ratio is highly misleading in light of the fact that they

- Neglected to publish the absolute liver weights of the animals and,
- The animals' bodyweight gain in the groups receiving OEP-NF was clearly suppressed relative to the vehicle control group.

Rather than report the absolute liver weight, the authors attempt to conflate a change in the liver to body weight ratio as an indication that liver hypertrophy occurred, when they, quite unusually, provide no evidence in the form of absolute liver weight data for the reader [72,82]. The liver to bodyweight ratio would normally be an acceptable measure of liver hypertrophy, except in a case where the animals are losing bodyweight or more precisely, gaining bodyweight at a lower rate than the vehicle control, which appears to be what occurred in this study. While the authors also do not provide body weight change data for the reader, if one evaluates the "bodyweight dynamics" reported for the animals in graphs, it becomes clear that the animals fed low and high doses of OEP-NF, gained much less bodyweight relative to the vehicle control [72]. This should not be surprising, considering OEP-NF was intended for weight loss. Clearly, the authors were aware that the product is intended to cause weight loss, thus their neglect to report the absolute liver weight and body weight change is unacceptable [72]. Looking at the published graphs, one can see that the CD-1 males and females, along with the B6C3F1 males and females all had suppressed body weight gain relative to the controls, thus even in those whose liver weights stayed the same, a significant decrease in bodyweight would lead to an elevated liver to bodyweight ratio [5].

Such issues with using only the liver to body weight ratio have been noted as far back as 1976 by Stevens, who pointed out the flaw in using the organ to bodyweight ratio while neglecting to consider the absolute weight of the organ, in animals that have lost bodyweight [83]. Takizawa further noted this issue and Andersen, et al., have since suggested a multivariate analysis of variance (MANOVA) to account for such issues [84,85].

There are also examples of such occurrences with natural products. Nukitrangsan, et al., sought to determine the potential for a commonly consumed plant in Japan, *Peucedanum japonicum*, to suppress body weight gain in mice as

a method for treating obesity [86]. Indeed, the plant did cause suppressed body weight gain in the study, confirming the anti-obesity potential of the plant [86]. However, despite not finding any indications of toxicity in the mice, there was an increase in the liver to body weight ratio. The authors, however, correctly point out that this was simply due to the fact that the body weight of the animals had decreased, while the liver weight stayed the same and thus was not an indicator of liver enlargement or toxicity.

Inappropriate study design for evaluation of synergistic, additive or potentiating effects: Yet another issue with the design of this study is that, according to the authors, only one of the four formulas was apparently considered for evaluation, despite all being implicated, each with varying ingredients, including one which lacked aegeline and instead contained DMAA. If as Miousse, et al., hypothesize, the supposed hepatotoxicity was due to a combination of these specific ingredients, one must explain why a formulation that did not possess that combination is also able to do so, despite for example, multiple lines of evidence demonstrating a lack of hepatotoxic potential with DMAA and DMAA-containing formulas [See Part 1] [5].

More importantly, the design of the study itself neglects to actually confirm what Miousse, et al., hypothesize [5]. That is, they believe that not one ingredient, but the combination of ingredients can cause toxicity. Yet, to demonstrate such an assertion, one must not only study a combination of ingredients, but each individual ingredient itself to truly demonstrate that the effects are not due to a single ingredient or that there is truly some sort of "synergistic", "additive" or "potentiating" effect [44,87]. Specifically, there should have been a caffeine; higenamine; aegeline; yohimbine; and control group which would then have been compared to the total formula, OEP-NF. Yet, inexplicably this was not done. In contrast, a randomized, controlled trial was conducted in a group of 48 normal, healthy men who were assigned to receive a placebo (n=12), caffeine (n=12), higenamine (n=12), or the combination of caffeine, higenamine, and yohimbine (n=12) over an 8-week period. Amongst other variables assessed for safety, liver health/function was assessed after 4 and 8-weeks of administration. The results demonstrated no significant change from baseline or between groups for bilirubin, alkaline phosphatase, AST, ALT, and GGT, demonstrating a lack of any harmful interactions amongst ingredients in OEP-NF [19].

The authors conflate the controversial combination of ephedra or ephedrine and caffeine with their claim of an unknown interaction in OEP-NF. Yet, there are key distinctions to be made. As already noted, unlike this study by Miousse, et al., those evaluating the potential synergistic or additive effects of caffeine and ephedra or ephedrine compared the combination with the single ingredients to determine if indeed there was a true effect, as it was done in the case of the combination of caffeine, higenamine, and yohimbine [19,88,89]. Furthermore, ephedrine and caffeine were studied for cardiovascular toxicity, not hepatotoxicity. Last, in the case of ephedra or ephedrine and caffeine, the molecular mechanisms through which they exert their synergistic or additive effects are well-known (i.e., cAMP modulation via G-protein-coupled receptors and adenosine antagonism, respectively [90,91]. Miousse, et al., have given no identification or a reasonable hypothesis as to how this occurs with OEP-NF [5]. Furthermore, as already noted the combination of 3 out of 4 of the ingredients in OEP-NF (i.e., caffeine, higenamine, and yohimbine) showed no evidence of any synergistic, additive or potentiating adverse effects [19].

Inaccurate interpretation of ALT/AST and miR-122 values: With respect to the increase in liver enzymes, as an example of what the authors refer to are the modest changes in ALT/AST at week 13 in the B6C3F1 female mice only (i.e., no changes were noted in the male B6C3F1 mice at week 13 [5], nor at the 4 week mark in either male or female B6C3F1 mice nor any of the CD-1 mice at week 4 or 13), despite being statistically significant are not considered to be a clinically or toxicologically significant effect. This is because, as the HED Toxicology Science Advisory Council (a scientific advisory council which advises the US EPA in the Office of Pesticide Programs, Health Effects Division) has noted, regarding changes in values of ALT and AST, *"Statistical significance, by itself, is not a reliable indication of liver toxicity. Among the factors to take into consideration are: laboratory variability; the normal range for each parameter in the species and strain at that laboratory; study to study variability; variability due to the number of animals per group; variability of enzyme levels as a function of age; possible presence of isoenzymes; and dose-response concordance*" [92]. It appears the authors of Miousee, et al., neglected to consider any of this [5].

The published authorities and regulatory agencies have also noted that "*It is common with hepatocellular hypertrophy to get some increase in ALT or AST with no evidence of hepatic injury, therefore increases should not be considered adverse until they are at least 2-fold to 3-fold greater than control levels". Below that level, they are usually within*

the normal range for the animal. In extreme cases, it is not unusual to observe an order of magnitude increase or greater" [62,63,92].

Indeed, while the increases in ALT and AST seen in the female B6C3F1 mice at week 13 were statistically significant, they were also in fact below a 2-fold increase greater than the control levels and thus are considered non-adverse [5]. Furthermore, the authors make only vague statements and present no corroborating evidence of an effect upon any histopathological variable for the 13-week female B6C3F1 mice receiving the 1x and 3x dose (nor any other groups of mice) [72].

Evaluating the conclusions from the 3rd International European Society of Toxicologic Pathology (ESTP), which convened a panel of experts from around the world, the authors provide a weight-of-evidence approach to distinguish between adverse and adaptive (i.e., non-adverse/not harmful and of little relevance to humans) effects upon the liver of experimental animals [81]. This weight of evidence approach is the standard used by toxicologists to determine whether there is evidence to support potential hepatotoxic effects in humans based upon animal studies. It states that in the absence of histological changes (i.e., the authors present no actual evidence of histological effects at a greater rate than vehicle control) a "weight-of-evidence approach" should be used to determine if there is evidence of hepatocyte damage. The expert working group asks the reader if, "*there is clinical pathology evidence of hepatocyte damage characterized by a dose-dependent and biologically significant and consistent increase in at least two liver parameters*" [81]:

- At least x2 to x3 increase in ALT.
- A biologically significant change in other biomarkers of hepatobiliary damage (ALP, AST, GGT, GLDH, etc.).
- A biologically significant change in another clinical pathology marker indicating liver dysfunction (albumin, bilirubin, bile acids, coagulation factors, cholesterol, triglycerides, etc.).

The authors then state that "*clinical pathology changes should corroborate each other, be consistent with the expected species-specific patterns of change resulting from hepatobiliary injury, and take into account target pharmacology that may non-adversely alter one or more of these biomarkers. It should be noted that statistical significance alone is not a reliable indicator of hepatic toxicity*" [81].

As noted, the increase in ALT/AST at week 13 in the B6C3F1 female mice (i.e., no changes were noted in the male B6C3F1 mice at week 13, nor at the 4 week mark in either male or female B6C3F1 mice nor any of the CD-1 mice at week 4 or 13) [5], while being statistically significant, was below a 2-fold increase greater than the control group. Thus, the first bullet point is negative as is the second (i.e., AST was below a 2-fold increase beyond control and only increased in the female B6C3F1 mice at week 13) [81]. This also speaks to consistency as one should have seen results that occurred throughout the study and corroborated rather than contradicted one another [81]. For the final bullet point, the authors did not bother to measure another clinical pathology marker indicating liver dysfunction as listed by the expert group (e.g., bilirubin, albumin, coagulation factors, bile acids) [81]. However, even if one used miR-122, whose relevance is questionable, particularly if the formula being evaluated was intended to stimulate the release of free fatty acids (i.e., lipolysis), only the male CD-1 mice, at week 4 and only with the 3x dose, experienced a statistically significant increase in miR-122 (i.e., the male CD-1 mice experienced a seemingly dose-dependent decrease in miR-122 in the 13-week study, while the week 4 female CD-1 mice experience a decrease as well), yet these same mice experienced no increase in AST or ALT; furthermore, the authors offer no actual evidence of histopathological changes to the liver [72]; the female CD-1 mice at week 4 and 13 experienced no change in miR-122 (in fact, the 1x and 3x dose groups were lower than vehicle control), nor did the male and female B6C3F1 mice at weeks 4 and 13 (with the exception of the 13-week female B6C3F1 mice receiving 1x OEP-NF, but not the 3x dose). Additionally, and just as importantly, when considering what few changes were noted, they were not "consistent, biologically significant or dose-dependent" [81]. Instead, the only group of mice in the entire 4 and 13-week study groups that showed a change in ALT/AST after repeated OEP-NF administration was the 13-week female B6C3F1 mice [5]. Yet, these changes were not biologically/toxicologically significant as they were below the 2 to 3-fold increase beyond control. The only groups in the entire study that showed an increase in miR-122 after repeated consumption of OEP-NF were the male CD-1 mice and only at week 4 given the 3x dose (the 13-week male CD-1 showed a clear decrease in miR-122 with the 1x and 3x dose compared to vehicle control) and the female B6C3F1 mice at week 13 given the 1x dose, but not the 3x dose, which was actually lower than control values [5]. Thus,

one can easily answer "no" to all 3 of these questions, which, according to the expert working group, indicates that "increases in liver organ weight and liver cell hypertrophy due to enzyme induction can be considered as an adaptive response to a xenobiotic and of little relevance to man". Yet, since the authors present only the liver to body weight ratio (i.e., rather than actual liver weights) for animals that clearly showed a reduced rate of weight gain relative to vehicle control animals, it is questionable whether the weight of evidence approach is even needed.

A Post-hoc Bioassay for "Genuine Responders": Miousse, et al., apparently conducted a post-hoc analysis of individual data, portraying individual mice as being, "genuine responders". Yet, there are several critical flaws with this approach. First, there is no *in vivo* bioassay known to determine or predict idiosyncratic liver toxicity or more specifically, "genuine responders" as the authors claim; rather, this appears to be a case of data dredging, albeit without actual statistical comparisons.

The authors go through the individual data for each mouse in the 4 and 13-week studies and assign an arbitrary notation of "responder phenotype", which they indicate is a significant change in that particular variable (i.e. liver to body weight ratio, ALT, AST, and miR-122) that is greater than two standard deviations (SD) from the mean of the vehicle control. First, while outlier analyses can be useful, the use of two standard deviations as opposed to three standard deviations to define an outlier is unusual. While one can in some circumstances use a lower SD than 3 to search for outliers, it must be based upon the distribution of the data at hand. In this case, the indication by the authors that even the vehicle control group had outliers using two SD indicates that it is not an appropriate choice; actual screens designed for detecting hepatotoxicity in vitro typically use 3 SD [93,94].

It appears the authors are attempting to use a multi-parameter model employed with *in vitro* assay based toxicity screens, in which two or more positive values are considered a "hit" or an indicator of hepatotoxicity. Yet, the differences between what Miousse, et al., have attempted and such methods are stark [5]. For example, in a paper by Sirenko, et al., which used a model similar to what Miousse, et al., apparently attempted, albeit *in vitro*, the values are not for individual samples (or in this case individual mice), but are the mean or averages of the group, which is of course necessary as statistical comparisons are made between the means or averages of groups in order to determine whether changes, if any, are due to mere chance/randomness or a real effect [5,94]. By analyzing individuals, one is taking single data points that may vary, sometimes greatly, due to myriad variables, including natural variations between individuals or differences in responses to the excessive doses of caffeine and potentially yohimbine [95]. The wide variation in clinical markers of liver function/toxicity is in fact noted by expert working groups when noting that statistical significance alone should not be construed as evidence for a significant change [81,92]; yet, the analysis by Miousse, et al., is not even a statistical analysis [5].

Even ignoring the flaw of using individual data instead of mean or averages for a group, the authors would need to have also included positive and negative controls as Sirenko, et al., have done [94]. Negative and positive controls are vital to determine the validity of the assays by calculating the specificity, sensitivity and predictive value.

Additionally, the Sirenko, et al., study and assays like it are intended to evaluate single compounds, not mixtures like that in OEP-NF [94]. In order for this group to have a valid assay, aside from the aforementioned issues, they would have needed to use each individual compound in the mixture as controls in order to ascertain whether any of the individual components are responsible for the effects seen with the total mixture [87]. Indeed, due to the incorrect dose extrapolation performed by the group, especially regarding caffeine and potentially yohimbine, this would be especially important.

Lack of Transparency for Designation of "Outliers": Along with the lack of providing the body weight data, absolute liver weight data and histopathological data, the authors yet again do not include the individual data for these mice so that others may evaluate their claims of "outliers" [72].

Incorrect Claim of Dose-Dependency: Contrary to the authors' claims, there was no dose-dependency in the studies as such an evaluation requires the use of a low, intermediate and high-dose group, along with a control [96-99]. Dose-dependency typically appears as a tiered rise in each group with the low-dose group being higher (or lower depending upon the directional effect of the variable), the intermediate being greater than the low-dose group and the high-dose group being greater than the intermediate; each group may be statistically significant from one another and the control group, although this may not always be the case. Even evaluating the graphs provided by the authors, one can see that in many cases, there is a decline in a given marker as the dose increases (e.g., ALT and miR-122 values in CD-1

male 13-week mice) [5], no change or a decrease in the low dose group followed by a rise in the high dose group (e.g., AST and miR-122 CD-1 female 13-week mice) or paradoxically [5], a rise in the low dose group but no rise or even a decline in the high dose group (e.g., ALT/AST for B6C3F1 male 13-week mice and AST along with miR-122 for CD-1 male 13-week mice and miR-122 for B6C3F1 female 13-week mice) [5]. Further, the moderate and inconsistent increase in miR-122 also was likely due to its active release rather than any passive release from damaged hepatic tissue considering the authors offer no corroborating data to demonstrate actual liver injury or any indication of any dose-dependency in this regard. Of course, the toxic doses of caffeine administered only confound interpretation further. It is likely that the modest, discordant and inconsistent elevations in ALT/AST were from non-hepatic sources or due to multi-organ caffeine (and potentially yohimbine) toxicity due to the excessive doses used in the animals.

Incorrect claims regarding effects upon gene expression: Miousse, et al., the next attempt to evaluate the hepatotoxicity of OEP-NF by using gene expression assays. However, as it has been pointed out by Chen, et al., gene expression assays are not a reliable method for determining potential hepatotoxicity, noting that: "*Additionally, the transcriptomic approaches have been for endpoints not necessarily relevant to DILI, that is, the measured altered gene expression might or might not be associated with mechanisms involving hepatocellular injury*" [100].

Ahktar similarly points out that humans and mice differ dramatically in gene expression in the liver stating: "*Wide differences have also become apparent in the regulation of the same genes, a point that is readily seen when observing differences between human and mouse livers. Consistent phenotypes (observable physical or biochemical characteristics) are rarely obtained by modification of the same gene, even among different strains of mice. Gene regulation can substantially differ among species and may be as important*" [2].

As it will be shown here, the reliability of these assays as indicators of hepatotoxicity is questionable when extrapolating from animals to humans as the directional changes may be reversed and as importantly, as Ahktar notes the resultant phenotypes are rarely consistent between species or even between strains of mice [2]. Miousse, et al., attempt to imply that any change in gene expression indicates that hepatotoxicity is occurring and that it is relevant to humans, which is misleading [5]. First, Miousse, et al., address the fact that cluster of differentiation 36 or CD36 was up-regulated in the CD-1 and B6C3F1 mice, referencing a study where CD36 has been shown to inhibit hepatic lipid accumulation [5,101]. Miousse, et al., further state that in the same study, the down-regulation of CD36 was responsible for a hepatoprotective effect [5,101], effectively arguing that an increase in CD36 is an indicator of hepatotoxicity and that this supposed paradoxical effect between the Zheng, et al., study and what occurred in the mice given the OEP-NF formula is a clear sign that multi-ingredient formulas can have drastically different effects compared to their single components [5,101]. Unfortunately, Miousse, et al., neglect to consider that the Zheng, et al., study was conducted in zebrafish larvae, which are not even mammals [101]. Furthermore, the authors ignore the fact that CD36 has already been shown in mammals such as mice and humans to be up-regulated in response to caffeine and exercise as well as in HepG2 cells, with CD36 likely playing a role in the fatty acid oxidation seen after caffeine administration [102-105]. Thus, confirming the warnings of others, and contrary to what the author's claim, the increase in CD36 in mice after being given a supplement containing caffeine, which was also designed to increase fatty acid oxidation, is not surprising nor an indication of hepatotoxic potential in humans [100].

The authors also note that mice given OEP-NF showed increased expression of thioredoxin reductase 1 (TXNRD1) and NAD(P)H quinone dehydrogenase I (NQO1); these are known protective antioxidant enzymes which are increased in response to foods such as broccoli and other cruciferous vegetables as well as fruits [106-111]. In essence, the authors attempt to conflate mere changes in the expression of these genes with toxicity, while neglecting to mention that the directional changes associated with liver toxicity are overwhelmingly in the opposite direction as that seen with OEP-NF, while further neglecting to address that at least some of these changes are known to occur with hepatoprotective substances.

The authors also cite a study by Choi, et al., which showed that berberine increased CD36 expression which was tied to hepatic steatosis [112]. The Choi, et al., the study is irrelevant for several reasons. First, as noted previously, caffeine alone has been shown to increase CD36 in human liver cells [112]. Second, the study cited by Miousse et, al., concerns berberine potentially causing steatosis in rodents [5]. However, steatosis is fatty accumulation in the liver which is not the same as hepatocellular injury (i.e., what is claimed to have happened in cases of OEP-NF associated hepatotoxicity); in fact, despite the elevation of CD36 in the mice given berberine in the study cited, there were

no actual indications of liver injury as ALT, GGT and bilirubin remained unchanged, while AST which increased, remained within the normal range, indicating that the increase was likely from an organ other than the liver [112]. Interestingly, the authors of the Choi, et al., study, seem to take note of several issues which should have been noticed by Miousse, et al., in their own study, including depressed bodyweight's effect upon the liver to body weight ratio and an extrahepatic rise in transaminase [5,112].

Even without considering the above, the authors' citation of this study showing berberine induces CD36 expression in mice, actually undermines their argument. For example, if berberine's increase in CD36 were, in fact, a hepatotoxic modulation, one should then very easily find cases of berberine-induced hepatotoxicity. To the contrary, despite berberine's use in China for centuries, there are no reports of berberine causing hepatocellular injury in humans in the scientific literature. Rather, berberine has been given to humans with preexisting liver injury and not only failed to cause harm but was actually determined by the authors to have improved liver function by reducing ALT and AST values [113]. In addition, there are dozens of published papers demonstrating that berberine is actually hepatoprotective; in fact, it has even outperformed silymarin (a compound often used to treat xenobiotic-induced liver toxicity) and n-acetyl l-cysteine (NAC; a gold-standard treatment for acetaminophen toxicity) [114-117]. In the case of NAC, berberine actually produced greater hepatoprotective effects in rodents given acetaminophen [117].

Additionally, to show the incongruent nature of such arguments by Miousse, et al., a study in humans has actually evaluated the effect of berberine for its ability to treat fatty liver or steatosis (NAFLD or nonalcoholic fatty liver disease) and found that it lowered the hepatic fat content of the liver more than a standard treatment [5,118]. Furthermore, a meta-analysis which examined all of the published studies in humans evaluating the effect of berberine upon fatty liver in humans showed that it, in fact, reduced hepatic fat, not increased it [119]. Thus, while Miousse, et al., error by citing the Choi, et al., study for multiple reasons mentioned; human data yet again corroborate the warnings of others showing effects in mice which fail to translate to humans and in fact, show just the opposite, with berberine being used successfully as a treatment for hepatic steatosis [2,5,100,112].

Miousse, et al., cite studies which they claim support the notion that increased NQO1, a well-established antioxidant, and cytoprotective enzyme, is an indicator of liver damage [5]. The first study they reference is Aleksunes, et al., which did indeed find that NQO1 was increased in human livers that were damaged by acetaminophen [120]. However, the authors are citing studies in humans and conflating them with the results found in mice which have shown with berberine and CD36, is an ill-founded approach and corroborates the warnings of others when interpreting such data in mice and applying it to humans [2,100]. Most importantly, the authors miss a critical point; OEP-NF did not increase NQO1 in humans, rather, the study by Miousse, et al., was conducted in mice [5]. In mice and rodents in general, NQO1 is increased in liver tissue in response to healthful foods like cruciferous vegetables such as broccoli, fruit and vegetable juice, garlic and onions, the dietary ingredient curcumin, resveratrol, lycopene, astaxanthin, blueberry extract and black currant [109-111,121,122-124]. NQO1 is in fact, considered an antioxidant enzyme which is thought to be responsible for the cancer preventative effects seen with foods such as cruciferous vegetables.

Miousse, et al., cite a paper from Palming, et al., erroneously claiming that they report elevated levels of NQO1 in the liver correlating with the clinical markers of liver dysfunction [5,125]. However, Palming, et al., measured NQO1 expression, not in liver tissue, but subcutaneous fat, thus it is of questionable relevance [125]. Furthermore, OEP-NF increased NQO1 in mice, not humans, thus logically; one should look to mice for examples of what increased NQO1 causes. For example, in mice, the known hepatotoxic drug, acetaminophen decreased NQO1, while the antioxidant and dietary ingredient, quercetin, increased NQO1, causing a hepatoprotective effect which was at least in part, mediated by the restoration of the NQO1 [126]. If NQO1 is increased by non-hepatotoxic and in fact, hepatoprotective substances in mice and it is decreased by known hepatotoxicants, one might rightfully point out that OEP-NF, if anything, shows evidence of a hepatoprotective effect, not hepatotoxicity. This is consistent not only with the ingredients such as aegeline [8] but the anti-oxidative effects shown by Zovico, et al., in rats given OEP [127]. Nonetheless, just as the case of the berberine study, the type of liver dysfunction discussed in the Palming et al. [125] paper was hepatic steatosis or fatty liver, which is a complication seen in human obesity. Fatty liver is in no way the same as hepatocellular injury as seen in cases of HILI/DILI; not a single case of alleged harm from OEP-NF in humans has been a case of the induced fatty liver.

The authors also mention that OEP-NF affected fructose-bisphosphate aldolase (ALDOA); that is, it was increased.

However, interestingly, ALDOA expression was shown to decrease in CD-1 mice administered the triazole fungicides which Miousse, et al., reference when claiming an apparent association between CD36 and hepatotoxicity [5,128]. Thus, OEP-NF had the opposite effect of those fungicides. Other examples which actually show the opposite of what the authors claim can be found by looking at MLX interacting protein-like (Mlxipl) and Cell Division Cycle 14B (Cdc14b), which have been shown to decrease in the livers of mice after experiencing necrosis from acetaminophen [129]. Conversely, OEP-NF increased expression of these genes [5].

A claimed mechanism which is unsupported: The authors also apparently attempt to indicate that a potential mechanism for the supposed hepatotoxicity of OEP-NF is through a biphasic dose-response like that seen with substances such as Epigallocatechin gallate (EGCG), where anti-oxidant effects are noted at low doses and pro-oxidant doses are seen with higher doses. However, unlike aegeline and the ingredients in OEP-NF, EGCG, in particular, has demonstrated toxicity to normal cells as well as the cancerous hepatic cells (HEPG2) in non-MTT based assays, at concentrations as little as 100 µmol and has shown dose-dependent indications of hepatotoxicity in animal models [8,130-134]. In mice, for example, EGCG was shown to cause hepatotoxicity, producing moderate to severe necrosis of the liver while causing well over 100-fold increases in ALT (i.e., 138-184-fold), as opposed to the lack of any noted necrosis in the Miousse, et al., study and the inconsistent but moderate (i.e., increases within the normal range, below the 2-3x threshold for toxicological significance) increases in ALT and AST [5,133]. Of course, it should also be noted once again that Miousse, et al., also administered doses of caffeine alone that were toxic [5]. Unlike the ingredients in OEP-NF, EGCG has also been shown to produce pro-oxidative effects at high concentrations, while demonstrating the ability to inhibit major antioxidant enzymes in hepatic tissue at toxic doses, while simultaneously activating Nrf-2 and at even higher, lethal doses, inhibiting both antioxidant enzymes as well as Nrf-2 [135]. Miousse, et al., also apparently indicate that any activation of Nrf-2, if prolonged could lead to hepatic injury [5]. Yet, compounds in broccoli, curcumin and the recently FDA-approved drug dimethyl fumarate, are all known Nrf-2 activators which have not been shown to cause liver toxicity, to the contrary a broccoli extract has shown success in treating liver dysfunction in humans, while curcumin has been used to treat non-alcoholic fatty liver disease in humans [136-145]. Furthermore, coffee and even caffeine itself at 15 mg/kg were shown in rats to activate Nrf-2 as well [146,147]. Thus, the activation of Nrf-2 and its potential role in liver protection versus liver toxicity may depend at least to some extent upon how it is activated and the toxicity of the molecule targeting Nrf-2, either directly or indirectly.

Oddly, the authors also comment that the OEP-NF did not cause weight loss, however, in animals that are growing in bodyweight as evidenced from the control group weight gain; the reduction in weight gain is typically noted with agents that may help with weight loss [43].

CONCLUSION

In summary, the Miousse, et al., the study is replete with numerous design and interpretational errors by the authors. The authors performed inappropriate dose extrapolation of caffeine and likely yohimbine as well and didn't follow basic guidelines for assessing toxicity and hepatotoxicity in animal models while failing to provide evidence for actual toxic effects upon the liver. More careful and appropriate study design, methodology and adherence to basic and accepted practices for study design and interpretation would prevent such occurrences.

DECLARATIONS

Conflict of Interest

The author has served as a consultant to USPlabs, the manufacturer of OxyELITE Pro and is a defendant in ongoing litigation related to OxyELITE Pro. USPlabs was not involved in the conception, writing, or editing of this article. The views expressed here are those of the author.

REFERENCES

[1] Pound, Pandora, and Michael B. Bracken. "Is animal research sufficiently evidence-based to be a cornerstone of biomedical research?." *BMJ: British Medical Journal*, Vol. 348, 2014.

[2] Akhtar, Aysha. "The flaws and human harms of animal experimentation." *Cambridge Quarterly of Healthcare Ethics*, Vol. 24, No. 4, 2015, pp. 407-19.

[3] Hackam, Daniel G., and Donald A. Redelmeier. "Translation of research evidence from animals to humans." *Journal of the American Medical Association*, Vol. 296, No. 14, 2006, pp. 1727-32.

[4] Mak, Isabella WY, Nathan Evaniew, and Michelle Ghert. "Lost in translation: animal models and clinical trials in cancer treatment." *American Journal of Translational Research*, Vol. 6, No. 2, 2014, p. 114.

[5] Miousse, Isabelle R., et al. "Safety assessment of the dietary supplement OxyELITE™ Pro (New Formula) in inbred and outbred mouse strains." *Food and Chemical Toxicology*, Vol. 109, 2017, pp. 194-209.

[6] Arseculeratne, Sarath N., A. Leslie Gunatilaka and Ralph G. Panabokke. "Studies on medicinal plants of Sri Lanka. part 14: toxicity of some traditional medicinal herbs." *Journal of Ethnopharmacology*, Vol. 13, No. 3, 1985, pp. 323-35.

[7] Klontz, Karl C., et al. "The role of adverse event reporting in the FDA response to a multistate outbreak of liver disease associated with a dietary supplement." *Public Health Reports*, Vol. 130, No. 5, 2015, pp. 526-32.

[8] Singh, Abhilasha, et al. "Aegeline vs Statin in the treatment of Hypercholesterolemia: a comprehensive study in rat model of liver steatosis." *Functional Foods in Health and Disease*, Vol. 8, No. 1, 2018, pp. 1-16.

[9] Mohammed, Magdy M., et al. "Two new cytotoxic furoquinoline alkaloids isolated from *Aegle marmelos* (Linn.) Correa." *Natural Product Research*, Vol. 30, No. 22, 2016, pp. 2559-66.

[10] Galluzzi, Lorenzo, et al. "Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes." *Cell Death and Differentiation*, Vol. 16, No. 8, 2009, pp. 1093-107.

[11] Jaeschke, Hartmut, Mitchell R. McGill and Anup Ramachandran. "Pathophysiological relevance of proteomics investigations of drug-induced hepatotoxicity in HepG2 cells." *Toxicological Sciences*, Vol. 121, No. 2, 2011, pp. 428-30.

[12] Manda, V., et al. Pharmacokinetics of aegeline after oral administration in a mouse model. In: International Conference on the Science of Botanicals. Oxford, MS, 2017.

[13] Ramos, Sonia, et al. "Comparative Effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2)." *Journal of Agricultural and Food Chemistry*, Vol. 53, No. 4, 2005, pp. 1271-80.

[14] Syng-ai, Christine, A. Leela Kumari and Ashok Khar. "Effect of curcumin on normal and tumor cells: role of glutathione and bcl-2." *Molecular Cancer Therapeutics*, Vol. 3, No. 9, 2004, pp. 1101-08.

[15] Liang, Wei-Zhe, et al. "The effect of the phenol compound ellagic acid on Ca2+ homeostasis and cytotoxicity in liver cells." *European Journal of Pharmacology*, Vol. 780, 2016, pp. 243-51.

[16] Chen, Ching-Hsein, et al. "Synergistic anti-cancer effect of baicalein and silymarin on human hepatoma HepG2 Cells." *Food and Chemical Toxicology*, Vol. 47, No. 3, 2009, pp. 638-44.

[17] Waluga, Marek, et al. "Cardiovascular effects of ephedrine, caffeine and yohimbine measured by thoracic electrical bioimpedance in obese women." *Clinical Physiology*, Vol. 18, No. 1, 1998, pp. 69-76.

[18] Swann, Alan C., et al. "Norepinephrine and impulsivity: effects of acute yohimbine." *Psychopharmacology*, Vol. 229, No. 1, 2013, pp. 83-94.

[19] Bloomer, R. J., J. M. Schriefer and T. A. Gunnels. "Clinical safety assessment of oral higenamine supplementation in healthy, young men." *Human and Experimental Toxicology*, Vol. 34, No. 10, 2015, pp. 935-45.

[20] Blanchard, Otis L. and James M. Smoliga. "Translating dosages from animal models to human clinical trials-revisiting body surface area scaling." *The FASEB Journal*, Vol. 29, No. 5, 2015, pp. 1629-34.

[21] Sharma, Vijay and John H. McNeill. "To scale or not to scale: the principles of dose extrapolation." *British Journal of Pharmacology*, Vol. 157, No. 6, 2009, pp. 907-21.

[22] Wojcikowski, Ken and Glenda Gobe. "Animal studies on medicinal herbs: predictability, dose conversion and potential value." *Phytotherapy Research*, Vol. 28, No. 1, 2013, pp. 22-7.

[23] Rhomberg, Lorenz R. and Thomas A. Lewandowski. "Methods for identifying a default cross-species scaling factor." *Human and Ecological Risk Assessment*, Vol. 12, No. 6, 2006, pp. 1094-127.

[24] Kenyon, Elaina M. "Interspecies extrapolation." Computational Toxicology, edited by Reisfeld Brad, Mayeno Arthur N. Totowa, NJ: Humana Press, 2012, pp. 501-20.

[25] Glade, Michael J. "Caffeine-not just a stimulant." *Nutrition*, Vol. 26, No. 10, 2010, pp. 932-38.

[26] Lee, Sang-Rok, et al. "Acute oral intake of a higenamine-based dietary supplement increases circulating free fatty acids and energy expenditure in human subjects." *Lipids in Health and Disease*, Vol. 12, No. 1, 2013, p. 148.

[27] Blanchard, James and S.J.A. Sawers. "The absolute bioavailability of caffeine in man." *European Journal of Clinical Pharmacology*, Vol. 24, No. 1, 1983, pp. 93-98.

[28] Blanchard, James and Stewart J. A. Sawers. "Comparative pharmacokinetics of caffeine in young and elderly men." *Journal of Pharmacokinetics and Biopharmaceutics*, Vol. 11, No. 2, 1983, pp. 109-26.

[29] Beach, Cynthia A., Joseph R. Bianchine and Nicholas Gerber. "The excretion of caffeine in the semen of men: pharmacokinetics and comparison of the concentrations in blood and semen." *The Journal of Clinical Pharmacology*, Vol. 24, No. 2-3, 1984, pp. 120-26.

[30] Azcona, O., et al. "Evaluation of the central effects of alcohol and caffeine interaction." *British Journal of Clinical Pharmacology*, Vol. 40, No. 4, 1995, pp. 393-400.

[31] Arnaud, Maurice J. "Pharmacokinetics and metabolism of natural methylxanthines in animal and man." *Handbook of Experimental Pharmacology*, Vol. 200, 2011, pp. 33-91.

[32] Samojlik, Isidora, et al. "Pharmacokinetic herb-drug interaction between essential oil of aniseed (*Pimpinella anisum* L., Apiaceae) and acetaminophen and caffeine: a potential risk for clinical practice." *Phytotherapy Research*, Vol. 30, No. 2, 2015, pp. 253-59.

[33] Sakai, C., et al. "Species differences in the pharmacokinetic parameters of cytochrome P450 probe substrates between experimental animals, such as mice, rats, dogs, monkeys, and micro mini pigs, and humans." *Journal of Drug Metabolism and Toxicology*, Vol. 5, No. 6, 2014, p. 173.

[34] Palm, Paul E., et al. "Evaluation of the teratogenic potential of fresh-brewed coffee and caffeine in the rat." *Toxicology and Applied Pharmacology*, Vol. 44, No. 1, 1978, pp. 1-16.

[35] Hayes, A. Wallace and Claire L. Kruger. Hayes' Principles and Methods of Toxicology. Boca Raton, FL: CRC Press, 2014.

[36] Walum, E. "Acute oral toxicity." *Environmental Health Perspectives*, Vol. 106, No. 2, 1998, pp. 497-503.

[37] Zbinden, G. and M. Flury-Roversi. "Significance of the LD50-test for the toxicological evaluation of chemical substances." *Archives of Toxicology*, Vol. 47, No. 2, 1981, pp. 77-99.

[38] Latini, R., et al. "Dose-dependent kinetics of caffeine in rats." *Toxicology Letters*, Vol. 2, No. 5, 1978, pp. 267-70.

[39] Boyd, Eldon M. "The acute oral toxicity of caffeine." *Toxicology and Applied Pharmacology*, Vol. 1, No. 3, 1959, pp. 250-75.

[40] Boyd, Eldon M., et al. "The chronic oral toxicity of caffeine." *Canadian Journal of Physiology and Pharmacology*, Vol. 43, No. 6, 1965, pp. 995-1007.

[41] Wolf, Paul L. "Biochemical diagnosis of liver disease." *Indian Journal of Clinical Biochemistry*, Vol. 14, No. 1, 1999, pp. 59-90.

[42] Ozer, Josef, et al. "The current state of serum biomarkers of hepatotoxicity." *Toxicology*, Vol. 245, No. 3, 2008, pp. 194-205.

[43] Emmanuel, Agomuo, et al. "Effect of caffeine on some selected biochemical parameters using rat model." *Advances in Biology*, Vol. 2017, 2017, p. 9303276.

[44] Taiwo, Oluwakemi I. and Abdulfatai A. Adesokan. "Effects of high doses of exogenous taurine, caffeine, and taurine-caffeine combination on biochemical, hematological and histologic parameters of adult rabbits." *American Journal of Biochemistry*, Vol. 7, No. 4, 2017, pp. 63-72.

[45] Schilter, B., et al. "Guidance for the safety assessment of botanicals and botanical preparations for use in food and food supplements." *Food and Chemical Toxicology*, Vol. 41, No. 12, 2003, pp. 1625-49.

[46] Turnbull, Duncan, Joseph V. Rodricks and Gregory F. Mariano. "Neurobehavioral hazard identification and characterization for caffeine." *Regulatory Toxicology and Pharmacology*, Vol. 74, 2016, pp. 81-92.

[47] Turnbull, Duncan, et al. "Caffeine and cardiovascular health." *Regulatory Toxicology and Pharmacology*, Vol. 89, 2017, pp. 165-85.

[48] Banerjee, Priya, et al. "Fatal caffeine intoxication: a series of eight cases from 1999 to 2009." *Journal of Forensic Sciences*, Vol. 59, No. 3, 2014, pp. 865-8.

[49] Jabbar, Seema B. and Mark G. Hanly. "Fatal caffeine overdose: a case report and review of literature." *The American Journal of Forensic Medicine and Pathology*, Vol. 34, No. 4, 2013, pp. 321-4.

[50] Nawrot, P., et al. "Effects of caffeine on human health." *Food Additives and Contaminants*, Vol. 20, No. 1, 2003, pp. 1-30.

[51] Rudolph, T. and K. Knudsen. "A case of fatal caffeine poisoning." *Acta Anaesthesiologica Scandinavica*, Vol. 54, No. 4, 2010, pp. 521-3.

[52] Lee, William M. "Acetaminophen (APAP) hepatotoxicity-Isn't it time for APAP to go away?" *Journal of Hepatology*, Vol. 67, No. 6, 2017, pp. 1324-31.

[53] Valtin, Heinz. "Drink at least eight glasses of water a day." Really? Is there scientific evidence for "8 × 8"?" *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, Vol. 283, No. 5, 2002, pp. R993-1004.

[54] Farrell, D. J. and L. Bower. "Fatal water intoxication." *Journal of Clinical Pathology*, Vol. 56, No. 10, 2003, pp. 803.

[55] Gardner, John W. "Death by water intoxication." *Military Medicine*, Vol. 167, No. 5, 2002, pp. 432-4.

[56] Deichmann, W. B., et al. "What is there that is not poison? A study of the third defense by Paracelsus." *Archives of Toxicology*, Vol. 58, No. 4, 1986, pp. 207-13.

[57] Tam, S. William, Manuel Worcel and Michael Wyllie. "Yohimbine: a clinical review." *Pharmacology & Therapeutics,* Vol. 91, No. 3, 2001, pp. 215-43.

[58] Ho, A. K., et al. "Distribution and metabolism of tritiated yohimbine in mice*." Archives Internationales de Pharmacodynamie et de Therapie*, Vol. 194, No. 2, 1971, pp. 304-15.

[59] Hubbard, John W., et al. "The pharmacokinetic properties of yohimbine in the conscious rat." *Naunyn-Schmiedeberg's Archives of Pharmacology*, Vol. 337, No. 5, 1988, pp. 583-87.

[60] Owen, J. A., et al. "The pharmacokinetics of yohimbine in man." *European Journal of Clinical Pharmacology*, Vol. 32, No. 6, 1987, pp. 577-82.

[61] Wink, M., T. Schmeller and B. Latz-Brüning. "Modes of action of allelochemical alkaloids: interaction with neuroreceptors, DNA, and other molecular targets." *Journal of Chemical Ecology*, Vol. 24, No. 11, 1998, pp. 1881-937.

[62] Committee for Medicinal Products for Human Use. Guideline on Detection of Early Signals of Drug-Induced Hepatotoxicity in Non-Clinical Studies. London, UK: European Medicines Agency (EMEA/CHMP/SWP/150115/2006), 2006.

[63] Committee for Medicinal Products for Human Use. Reflection Paper on Non-Clinical Evaluation of Drug-Induced Liver Injury (DILI) EMEA. CHMP/SWP/150115/2006. London, UK: European Medicines Agency, 2010.

[64] Davies, Dave. "Review of the International Harmonisation of Clinical Pathology testing (IHCPT) recommendations on study design and clinical pathology testing." *Comparative Haematology International*, Vol. 5, No. 3, 1995, pp. 206-09.

[65] OECD. Section 4 (Part 408), Health Effects: Repeated Dose 90-Day Oral Toxicity Study in Rodents, Guideline for the Testing of Chemicals. Paris: Organisation of Economic Co-operation and Development, 1998.

[66] Chai, Chofit, et al. "A metabolic circuitry comprising free fatty acids, MIR-122 and triglycerides." *Journal of Hepatology*, Vol. 64, No. 2, 2016, p. S675.

[67] Church, Rachel J. and Paul B. Watkins. "The transformation in biomarker detection and management of drug-induced liver injury." *Liver International*, Vol. 37, No. 11, 2017, pp. 1582-90.

[68] Chai, Chofit, et al. "Metabolic circuit involving free fatty acids, microRNA 122, and triglyceride synthesis in liver and muscle tissues." *Gastroenterology*, Vol. 153, No. 5, 2017, pp. 1404-15.

[69] Zhang, Yanjie, et al. "Emerging role of microRNAs in mTOR signaling." *Cellular and Molecular Life Sciences*, Vol. 74, No. 14, 2017, pp. 2613-25.

[70] Aryal, Binod, et al. "MicroRNAs and lipid metabolism." *Current Opinion in Lipidology*, Vol. 28, No. 3, 2017, pp. 273-80.

[71] Redis, R. Simona and George A. Calin. "Snapshot: non-coding RNAs and metabolism." *Cell Metabolism*, Vol. 25, No. 1, 2017, p. 220.

[72] Maronpot, Robert R., et al. "Safety assessment and single-dose toxicokinetics of the flavoring agent myricitrin in Sprague–Dawley rats." *Food Additives and Contaminants: Part A*, Vol. 32, No. 11, 2015, pp. 1799-809.

[73] Murase, Takatoshi, et al. "Coffee polyphenols suppress diet-induced body fat accumulation by downregulating SREBP-1c and related molecules in C57BL/6J mice." *American Journal of Physiology-Endocrinology and Metabolism*, Vol. 300, No. 1, 2011, pp. 122-33.

[74] García-Segura, Laura, Martha Pérez-Andrade and Juan Miranda-Ríos. "The emerging role of microRNAs in the regulation of gene expression by nutrients." *Journal of Nutrigenetics and Nutrigenomics*, Vol. 6, No. 1, 2013, pp. 16-31.

[75] Rajan, Sujith, et al. "Aegeline inspired synthesis of novel β3-AR agonist improves insulin sensitivity *in vitro* and *in vivo* models of insulin resistance." *Metabolism*, Vol. 85, 2018, pp. 1-13.

[76] Satish, Sabbu, et al. "Aegeline inspired synthesis of novel amino alcohol and thiazolidinedione hybrids with antiadipogenic activity in 3T3-L1 cells." *European Journal of Medicinal Chemistry*, Vol. 143, 2018, pp. 780-91.

[77] Galitzky, J., et al. "α2-Antagonist compounds and lipid mobilization: evidence for a lipid mobilizing effect of oral yohimbine in healthy male volunteers." *European Journal of Clinical Investigation*, Vol. 18, No. 6, 1988, pp. 587-94.

[78] Lafontan, M., et al. "Alpha-2 adrenoceptors in lipolysis: α2 antagonists and lipid-mobilizing strategies." *The American Journal of Clinical Nutrition*, Vol. 55, No. 1, 1992, pp. 219-27.

[79] Harpaz, Eynav, et al. "The effect of caffeine on energy balance." *Journal of Basic and Clinical Physiology and Pharmacology*, Vol. 28, No. 1, 2017, pp. 1-10.

[80] Clarke, Joanna I., James W. Dear and Daniel J. Antoine. "Recent advances in biomarkers and therapeutic interventions for hepatic drug safety–false dawn or new horizon?" *Expert Opinion on Drug Safety,* Vol. 15, No. 5, 2016, pp. 625-34.

[81] Hall, Peter A., et al. "A Review of Adaptive (Adverse and Non-adverse) Changes-Conclusions from the 3rd International ESTP Expert Workshop." *Toxicologic Pathology*, Vol. 40, 2012, pp. 971-94.

[82] Sellers, Rani S., et al. "Society of toxicologic pathology position paper: organ weight recommendations for toxicology studies." *Toxicologic Pathology*, Vol. 35, No. 5, 2007, pp. 751-55.

[83] Stevens, M. T. "The value of relative organ weights." *Toxicology*, Vol. 5, No. 3, 1976, pp. 311-18.

[84] Takizawa, Tsuyoshi. "An unbiased comparison of organ weights when an inequality in body weight exists." *Toxicology*, Vol. 9, No. 4, 1978, pp. 353-60.

[85] Andersen, Helle, et al. "Multivariate statistical analysis of organ weights in toxicity studies." *Toxicology,* Vol. 136, No. 2-3, 1999, pp. 67-77.

[86] Nukitrangsan, Natthanan, et al. "Effect of *Peucedanum japonicum* thunb extract on high-fat-diet-induced obesity and gene expression in mice." *Journal of Oleo Science*, Vol. 61, No. 2, 2012, pp. 89-101.

[87] Bhat, A. Shakoor and Azad A. Ahangar. "Methods for detecting chemical–chemical interaction in toxicology." *Toxicology Mechanisms and Methods*, Vol. 17, No. 8, 2007, pp. 441-50.

[88] Brown, Christopher E., et al. "Combined effects of ephedrine-containing dietary supplements, caffeine, and nicotine on morphology and ultrastructure of rat hearts." *Journal of Caffeine Research*, Vol. 2, No. 3, 2012, pp. 123-32.

[89] Dunnick, J. K., et al. "Cardiotoxicity of ma huang/caffeine or ephedrine/caffeine in a rodent model system." *Toxicologic Pathology*, Vol. 35, No. 5, 2007, pp. 657-64.

[90] Astrup, Arne, et al. "Thermogenic synergism between ephedrine and caffeine in healthy volunteers: a double-blind, placebo-controlled study." *Metabolism*, Vol. 40, No. 3, 1991, pp. 323-29.

[91] Jones, Gareth. "Caffeine and other sympathomimetic stimulants: modes of action and effects on sports performance." *Essays In Biochemistry*, Vol. 44, 2008, pp. 109-24.

[92] HED Toxicology Science Advisory Council. Hepatocellular hypertrophy. HED Guidance Document #G0201. October, 2002.

[93] O'Brien, P. J., et al. "High concordance of drug-induced human hepatotoxicity with in vitro cytotoxicity measured in a novel cell-based model using high content screening." *Archives of Toxicology*, Vol. 80, No. 9, 2006, pp. 580-604.

[94] Sirenko, Oksana, et al. "High-content assays for hepatotoxicity using induced pluripotent stem cell-derived cells." *ASSAY and Drug Development Technologies*, Vol. 12, No. 1, 2014, pp. 43-54.

[95] Cumming, Geoff, Fiona Fidler and David L. Vaux. "Error bars in experimental biology." *The Journal of Cell Biology*, Vol. 177, No. 1, 2007, pp. 7-11.

[96] Sewell, Fiona, et al. "Opportunities to apply the 3Rs in safety assessment programs." *ILAR Journal,* Vol. 57, No. 2, 2016, pp. 234-45.

[97] Robinson, Sally, et al. Guidance on Dose Level Selection for Regulatory General Toxicology Studies for Pharmaceuticals. London, UK: NC3Rs/LASA, 2009.

[98] Parasuraman, S. "Toxicological screening." *Journal of Pharmacology and Pharmacotherapeutics*, Vol. 2, No. 2, 2011, pp. 74-79.

[99] The European Agency for the Evaluation of Medicinal Products. Evaluation of Medicines for Human Use. committee for Proprietary Medicinal Products (CPMP). London, UK: Note for Guidance on Repeated Dose Toxicity, 2000.

[100] Chen, Minjun, et al. "Toward predictive models for drug-induced liver injury in humans: are we there yet?" *Biomarkers in Medicine*, Vol. 8, No. 2, 2014, pp. 201-13.

[101] Zheng, Xinchun, et al. "Caffeine reduces hepatic lipid accumulation through regulation of lipogenesis and ER stress in zebrafish larvae." *Journal of Biomedical Science*, Vol. 22, No. 1, 2015, p. 105.

[102] Quan, H. Yan, Do Y. Kim and Sung H. Chung. "Caffeine attenuates lipid accumulation via activation of AMP-activated protein kinase signaling pathway in HepG2 cells." *BMB Reports*, Vol. 46, No. 4, 2013, pp. 207-12.

[103] Lally, J. S. V., et al. "Caffeine-stimulated fatty acid oxidation is blunted in CD36 null mice." *Acta Physiologica*, Vol. 205, No. 1, 2012, pp. 71-81.

[104] McFarlan, Jay T., et al. "*In vivo*, fatty acid translocase (CD36) critically regulates skeletal muscle fuel selection, exercise performance, and training-induced adaptation of fatty acid oxidation." *Journal of Biological Chemistry*, Vol. 287, No. 28, 2012, pp. 23502-16.

[105] Manio, M. Christian C., et al. "CD36 is essential for endurance improvement, changes in whole-body metabolism, and efficient PPAR-related transcriptional responses in the muscle with exercise training." *Physiological Reports*, Vol. 5, No. 10, 2017, p. e13282.

[106] Jeffery, Elizabeth H. and Marcela Araya. "Physiological effects of broccoli consumption." *Phytochemistry Reviews*, Vol. 8, No. 1, 2008, pp. 283-98.

[107] Hintze, Korry J., et al. "Induction of hepatic thioredoxin reductase activity by sulforaphane, both in Hepa1c1c7 cells and in male Fisher 344 rats." *The Journal of Nutritional Biochemistry*, Vol. 14, No. 3, 2003, pp. 173-79.

[108] Razis, A. F. Abdull, et al. "Intact glucosinolates modulate hepatic cytochrome P450 and phase II conjugation activities and may contribute directly to the chemopreventive activity of cruciferous vegetables." *Toxicology,* Vol. 277, No. 1-3, 2010, pp. 74-85.

[109] Razis, A. F. Abdull and Noramaliza M. Noor. "Cruciferous vegetables: dietary phytochemicals for cancer prevention." *Asian Pacific Journal of Cancer Prevention*, Vol. 14, No. 3, 2013, pp. 1565-70.

[110] Yuan, Linhong, et al. "Vegetable and fruit juice enhances antioxidant capacity and regulates antioxidant gene expression in rat liver, brain and colon." *Genetics and Molecular Biology*, Vol. 40, No. 1, 2017, pp. 134-41.

[111] Dinkova-Kostova, Albena T. and Paul Talalay. "NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1), a multifunctional antioxidant enzyme and exceptionally versatile cytoprotector." *Archives of Biochemistry and Biophysics*, Vol. 501, No. 1, 2010, pp. 116-23.

[112] Choi, You-Jin, et al. "Activation of AMPK by berberine induces hepatic lipid accumulation by upregulation of fatty acid translocase CD36 in mice." *Toxicology and applied pharmacology*, Vol. 316, 2017, pp. 74-82.

[113] Zhao, Wei, et al. "Reduction of blood lipid by berberine in hyperlipidemic patients with chronic hepatitis or liver cirrhosis." *Biomedicine and Pharmacotherapy*, Vol. 62, No. 10, 2008, pp. 730-31.

[114] Janbaz, K. H. and A. H. Gilani. "Studies on preventive and curative effects of berberine on chemical-induced hepatotoxicity in rodents." *Fitoterapia*, Vol. 71, No. 1, 2000, pp. 25-33.

[115] Zhao, Xiaoyan, et al. "Protective effects of berberine on doxorubicin-induced hepatotoxicity in mice." *Biological and Pharmaceutical Bulletin*, Vol. 35, No. 5, 2012, pp. 796-800.

[116] Domitrović, Robert, Hrvoje Jakovac and Gordana Blagojević. "Hepatoprotective activity of berberine is mediated by inhibition of TNF-α, COX-2, and iNOS expression in CCl4-intoxicated mice." *Toxicology*, Vol. 280, No. 1-2, 2011, pp. 33-43.

[117] Almani, S. Ahmed, et al. "Free radical scavenging activity of berberine in acetaminophen-induced liver injury." *International Journal of Surgery and Medicine*, Vol. 3, 2017, pp. 27-36.

[118] Yan, Hong-Mei, et al. "Efficacy of berberine in patients with non-alcoholic fatty liver disease." *PLoS One*, Vol. 10, No. 8, 2015, p. e0134172.

[119] Wei, Xiaoyun, et al. "The therapeutic effect of berberine in the treatment of nonalcoholic fatty liver disease: a meta-analysis." *Evidence-Based Complementary and Alternative Medicine*, Vol. 2016, 2016, p. 3593951.

[120] Aleksunes, Lauren M. "Up-regulation of NAD(P)H quinone oxidoreductase 1 during human liver injury." *World Journal of Gastroenterology*, Vol. 12, No. 12, 2006, p. 1937.

[121] Zhou, Yue, et al. "Dietary natural products for prevention and treatment of liver cancer." *Nutrients,* Vol. 8, No. 3, 2016, p. 156.

[122] Wu, Jia-Ching, et al. "Chemopreventive effect of natural dietary compounds on xenobiotic-induced toxicity." *Journal of Food and Drug Analysis*, Vol. 25, No. 1, 2017, pp. 176-86.

[123] Munday, Rex and Christine M. Munday. "Relative activities of organosulfur compounds derived from onions and garlic in increasing tissue activities of quinone reductase and glutathione transferase in rat tissues." *Nutrition and Cancer*, Vol. 40, No. 2, 2001, pp. 205-10.

[124] Iqbal, Mohammad, et al. "Dietary supplementation of curcumin enhances antioxidant and phase II metabolizing enzymes in ddY male mice: possible role in protection against chemical carcinogenesis and toxicity." *Pharmacology and Toxicology*, Vol. 92, No. 1, 2003, pp. 33-38.

[125] Palming, Jenny, et al. "The expression of NAD(P)H: quinone oxidoreductase 1 is high in human adipose tissue, reduced by weight loss, and correlates with adiposity, insulin sensitivity, and markers of liver dysfunction." *The Journal of Clinical Endocrinology and Metabolism*, Vol. 92, No. 6, 2007, pp. 2346-52.

[126] Truong, Van-Long, et al. "Quercitrin from *Toona sinensis* (Juss.) M. Roem. Attenuates acetaminophen-induced acute liver toxicity in HepG2 cells and mice through induction of antioxidant machinery and inhibition of inflammation." *Nutrients*, Vol. 8, No. 7, 2016, p. 431.

[127] Zovico, P. Vinicios, et al. "Effects of controlled doses of Oxyelite Pro on physical performance in rats." *Nutrition and Metabolism*, Vol. 13, No. 1, 2016, p. 90.

[128] Goetz, Amber K., et al. "Gene expression profiling in the liver of CD-1 mice to characterize the hepatotoxicity of triazole fungicides." *Toxicology and Applied Pharmacology*, Vol. 215, No. 3, 2006, pp. 274-84.

[129] Harrill, Alison H., et al. "Population-based discovery of toxicogenomics biomarkers for hepatotoxicity using a laboratory strain diversity panel." *Toxicological Sciences*, Vol. 110, No. 1, 2009, pp. 235-43.

[130] Saleh, Ibrahim G., et al. "Possible hepatocellular toxicity of EGCG under the influence of an inflammagen." *Journal of Applied Biomedicine,* Vol. 12, No. 4, 2014, pp. 291-99.

[131] Mezera, Vojtech, et al. "The effect of epigallocatechin gallate on hepatocytes isolated from normal and partially hepatectomized rats." *Canadian Journal of Physiology and Pharmacology,* Vol. 92, No. 6, 2014, pp. 512-17.

[132] Galati, Giuseppe, et al. "Cellular and *in vivo* hepatotoxicity caused by green tea phenolic acids and catechins." *Free Radical Biology and Medicine,* Vol. 40, No. 4, 2006, pp. 570-80.

[133] Lambert, Joshua D., et al. "Hepatotoxicity of high oral dose (-)-epigallocatechin-3-gallate in mice." *Food and Chemical Toxicology,* Vol. 48, No. 1, 2010, pp. 409-16.

[134] Saleh, Ibrahim G., et al. "Effect of green tea and its polyphenols on mouse liver." *Fitoterapia*, Vol. 90, 2013, pp. 151-59.

[135] Wang, Dongxu, et al. "Green tea polyphenol (-)-epigallocatechin-3-gallate triggered hepatotoxicity in mice: responses of major antioxidant enzymes and the Nrf2 rescue pathway." *Toxicology and Applied Pharmacology*, Vol. 283, No. 1, 2015, pp. 65-74.

[136] Kikuchi, Masahiro. "Sulforaphane-rich broccoli sprout extract improves hepatic abnormalities in male subjects." *World Journal of Gastroenterology,* Vol. 21, No. 43, 2015, p. 12457.

[137] Zhou, Yu, et al. "Broccoli seed extract: genotoxicity and subchronic toxicity studies." *Regulatory Toxicology and Pharmacology,* Vol. 73, No. 1, 2015, pp. 442-51.

[138] Alumkal, Joshi J., et al. "A phase II study of sulforaphane-rich broccoli sprout extracts in men with recurrent prostate cancer." *Investigational New Drugs,* Vol. 33, No. 2, 2014, pp. 480-89.

[139] Doss, Jennifer F., et al. "Phase 1 study of a sulforaphane-containing broccoli sprout homogenate for sickle cell disease." *PLoS One*, Vol. 11, No. 4, 2016, p. e0152895.

[140] Shapiro, Theresa A., et al. "Safety, tolerance, and metabolism of broccoli sprout glucosinolates and isothiocyanates: a clinical phase I study." *Nutrition and Cancer,* Vol. 55, No. 1, 2006, pp. 53-62.

[141] Cramer, Jenna and Elizabeth Jeffery. "Sulforaphane absorption and excretion following ingestion of a semi-purified broccoli powder rich in glucoraphanin and broccoli sprouts in healthy men." *Nutrition and Cancer,* Vol. 63, No. 2, 2011, pp. 196-201.

[142] Sharma, Ricky A., et al. "Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance." *Clinical Cancer Research*, Vol. 10, No. 20, 2004, pp. 6847-54.

[143] Cheng, Ann-Lii, et al. "Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions." *Anticancer Research*, Vol. 21, No. 4B, 2001, pp. 2895-900.

[144] Panahi, Yunes, et al. "Efficacy and safety of phytosomal curcumin in non-alcoholic fatty liver disease: a randomized controlled trial." *Drug Research*, Vol. 67, No. 4, 2017, pp. 244-51.

[145] Fox, Robert J., et al. "BG-12 (dimethyl fumarate): a review of mechanism of action, efficacy, and safety." *Current Medical Research and Opinion*, Vol. 30, No. 2, 2013, pp. 251-62.

[146] Salomone, Federico, Fabio Galvano and Giovanni L. Volti. "Molecular bases underlying the hepatoprotective effects of coffee." *Nutrients*, Vol. 9, No. 1, 2017, p. 85.

[147] Cavin, C., et al. "Induction of Nrf2-mediated cellular defenses and alteration of phase I activities as mechanisms of chemoprotective effects of coffee in the liver." *Food and Chemical Toxicology*, Vol. 46, No. 4, 2008, pp. 1239-48.