Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 131-145 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

8. Bioavailability and metabolism of maslinic acid, a natural pentacyclic triterpene

M. Emília Juan and Joana M. Planas
Departament de Bioquímica i Fisiologia and Institut de Recerca en Nutrició i Seguretat Alimentària (INSA-UB), Universitat de Barcelona (UB), Av. Joan XXIII 27-31, 08028 Barcelona, Spain

Abstract. Maslinic acid is a pentacyclic triterpene found in plants used in traditional medicine with antidiabetic, antitumor, antioxidant and parasitostatic activities. A diet rich in foods of plant origin could provide a regular supply of this bioactive compound since it has been detected in olives, spinaches, eggplants, chickpeas and pomegranates. The multiple biological effects elicited by maslinic acid suggest its putative use as nutraceutical. This review summarizes our study on the safety, pharmacokinetics, and metabolism after the oral administration of maslinic acid, conducted with the aim of increasing the knowledge about the bioavailability of this bioactive food component.

Introduction

The association between nutrition and human health is not a novel concept, since Ayurveda and Chinese medicine had already endorsed food as medicine, either as prevention for the development of certain ailments or as a treatment. This association was promoted by Hippocrates (460–377 BC) in

Correspondence/Reprint request: Dr. M.E. Juan. Departament de Bioquímica i Fisiologia, Universitat de Barcelona (UB), Av. Joan XXIII 27-31. 08028 Barcelona, Spain. E-mail: mejuan@ub.edu

his principle "Let food be thy medicine, and medicine be thy food". The relationship between nutrition and health has encouraged research on bioactive food components that could improve the physical and mental well-being and reduce the risk of disease. Among the different food from the diet, those from vegetable origin have been strongly associated with a reduced risk of developing chronic diseases, due to the presence of phytochemicals that are non-nutritive secondary metabolites. These bioactive compounds may have a lower potency if they are compared to drugs. However, phytochemicals could impact on health given that they are ingested regularly as part of the diet [1]. Different dietary molecules have been associated with physiological effects, such as glucosinolates, carotenoids, polyphenols and triterpenoids. Among the latter, stands out maslinic acid, which is currently in the early stages of the pre-clinical phase of research, as evidenced by the fact that the vast majority of published references mainly consisted of *in vitro* studies, with only a few studies conducted *in vivo*. Maslinic acid has been described to exert several biological activities, such as antidiabetic, antitumor, antioxidant and parasitostatic, among others [2]. Therefore, the present chapter focuses on the recent research in our group that has been aimed at assessing the safety of maslinic acid and at evaluating its bioavailability after oral administration.

1. Chemistry of maslinic acid

Maslinic acid or (2α,3β)-2,3-dihydroxyolean-12-en-28-oic acid (Fig. 1) was first isolated in 1927 from the leaves of *Crataegus oxyacantha* L. and was named "crategolic acid" [3].

The identification of this compound in *Olea europaea* L. came in 1961 when Caglioti *et al.* [4] found in olive husk a triterpenic compound with the same molecular formula and chemical structure as crategolic acid and termed it maslinic acid. The same year, Vioque and Morris [5] detected this pentacyclic triterpene in olive pomace. However, it was not until 1994 that Bianchi and coworkers [6] established that maslinic acid was the main pentacyclic triterpene in olives.

The biosynthetic pathway has been recently postulated in *Olea europaea* L. [7], where it has been suggested that sterols (primary metabolites) and non-steroidal triterpenoids (secondary metabolites) share 2,3-oxidosqualene as a common precursor. The cyclization of 2,3-oxidosqualene leads to the oleanyl cation as a precursor of erythrodiol, which is the first derivative of ß-amyrin. Further oxidation steps yield to the formation of

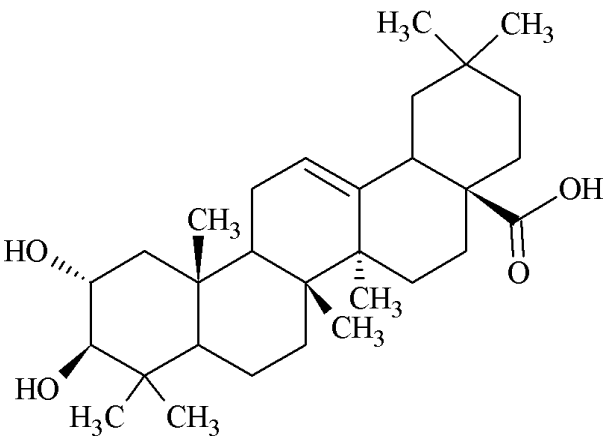

Figure 1. Chemical structure of maslinic acid.

oleanolic acid and its isomer maslinic acid. In the plant, maslinic acid acts as a phytoalexin that is a secondary metabolite involved in the protection against pathogens [8].

2. Sources of maslinic acid

Maslinic acid is broadly distributed in the plant kingdom, being detected in more than 30 species worldwide. This pentacyclic triterpene has been found in plants used in traditional medicine for the treatment of diverse affections. The leaves of banaba or *Lagerstroemia speciose* L. are especially rich in maslinic acid (4.96 ± 0.13 mg/g) [9]. The extracts of the banaba have been used for many years in folk medicine for the treatment of diabetes. *Crataegus monogyna* L., commonly known as hawthorn, contain 0.93 ± 0.07 mg/g of maslinic acid [9] and has been used as a remedy for the cardiovascular system given its hypotensive, antioxidant, anti-inflammatory, and vasodilating effects, among others. *Ortosiphon stamineus* L. possesses several pharmacological activities such as diuretic, hepatoprotective, antidiabetic, antihyperlipidemic, and has been described to have 0.84 ± 0.06 mg/g of maslinic acid [9]. Moreover, this pentacyclic triterpene has been described in *Eriobotrya japonica*, employed as antitussive and anti-inflammatory for chronic bronquitis as well as diuretic [10], *Geum japonicum* employed as diuretic [11], and *Agastache rugose* used in the treatment of intestinal disorders [12].

Maslinic acid has also been described in several edible plants that could provide a constant supply of this compound, especially in the countries bordering the Mediterranean Sea where the diet is rich in fruits and vegetables, olive oil is used for cooking as well as salad dressing and table olives are consumed as appetizer. The latter are a food with a particularly high content in maslinic acid although the concentration depend on the fruit variety and the method of elaboration. In fruits processed with natural fermentation, the concentrations of maslinic acid ranged from 1.32 ± 0.4 g/g fresh olive pulp in Kalamata to 0.28 ± 0.07 g/g fresh olive pulp in Manzanilla variety [13]. Virgin olive oil is obtained in a process involving pressing, which may disrupt the surface waxes on the fruit. Therefore, part of maslinic acid contained in the olive may be transferred to the oil. However, the amount of this pentacyclic triterpene in the oil is much lower than in the fruit, and depends on the oil quality. Extra virgin olive oil, with an acidity under 1% contains 64.2 ± 8.1 mg/kg of maslinic acid, an amount that increases to 193.9 ± 14.0 mg/kg in virgin olive oil, since the hydrolytic process that takes place during the extraction enables the release of this compound from the cuticular layer [14]. The presence of this compound has been reported in spinach (1260 mg/kg dry weight), eggplants (840 mg/kg dry weight), chickpeas (62 mg/kg dry weight), large lentils (40 mg/kg dry weight), and aromatic herbs such as basil (350 mg/kg dry weight) [15,16]. Maslinic acid has also been found in fruits, like kiwi (17.3 mg/kg), pomegranate (10.8 mg/kg), or mandarin (10.8 mg/kg) [17].

3. Toxicology

The beneficial effects on health described for maslinic acid granted the use of this compound as a possible nutraceutical. However, the safety of this bioactive compound needed to be assessed. Accordingly, the effect of high doses of maslinic acid on Swiss CD-1 mice were evaluated following the indications given by the Organization for Economic Cooperation and Development (OECD) guidelines [18] for the evaluation of preclinical acute and subacute toxicities. In first place, the effect of the single oral administration of maslinic acid was assessed by administering 1000 mg/kg to two adult Swiss CD-1 mice. The acute toxicity study was conducted in order to evaluate if the administration of a single high dose of this pentacyclic triterpene exerted any adverse effect on the animals over the immediately consecutive days. This evaluation of the acute toxicity showed that this pentacyclic triterpene did not have any negative effect on the

animals after the 15-day observation period in which body weight, food consumption as well as the general state of the animals was controlled, indicating that maslinic acid is practically nontoxic under these conditions [19].

In second place, the subacute toxicological evaluation was performed and consisted of the repeated oral administration for 28 days of maslinic acid at 50 mg/kg [19]. The dose selected corresponded to approximately 125 times the amount that may be consumed by a person eating 40 g or 10-medium sized olives and 33 g of olive oil a day. The results showed that no death or hazardous signs of toxicity were recorded during the experimental period. A decrease of a 13% was observed in the food intake of the treated mice compared to the controls, without affecting the body growth, which was equal in both groups [19]. A similar effect was indicated for the gilthead sea bream (*Sparus aurata*) that were fed with 100 mg/kg of maslinic acid for 210, reporting a decrease in the relative intake of diet 18% lower in the fish fed with the triterpene with respect to the control group and an increase of body weight in the treated group of only a 5% [20]. No effect on body weight was observed after the administration of 20, 40, and 80 mg/kg to juvenile dentex (*Dentex dentex*) during 49 days [21]. By contrast, maslinic acid has been described to stimulate body growth in healthy fish thus being proposed as a feed additive in aquiculture [22]. Rainbow trout (*Oncorhynchus mykiss*) fed with 25 and 250 mg/kg of maslinic acid in the diet during 225 days increased body weight, compared to the control group, in 19.3% and 29.2%, respectively. This effect was attributed to a higher growth rate of white muscle, which was correlated with hyperplasia and hypertrophy in the tissue [22].

The safety of maslinic acid was further evaluated by the assessment of hematology and clinical chemistry, which showed no differences between the control and the treated groups [19]. The absence of negative results obtained after the clinical chemistry evaluation corroborated that the treatment of mice with 50 mg/kg of maslinic acid did not compromise the overall health status of the animals since no alterations in metabolic processes in target organs were observed [19]. Worth mentioning is the point that maslinic acid did not have any significant effect on blood glucose in physiological conditions, despite being considered an anti-diabetic agent in different models of hyperglycemia [23,24]. It could be considered a positive outcome, since it would imply that maslinic acid could avoid the side effects caused by hypoglycemic treatments. No effects were observed on the lipid profile [19].

The lack of harmful effects exerted by the oral administration of 50 mg/kg of maslinic acid for 28 days was verified with a gross necropsy with the measurement of the organ weights and the histopathologic evaluation of the tissues [19]. The relative weight of liver, kidney, heart, lungs, and spleen were not different from controls. Remarkable the fact that no liver hyperplasia was observed in our study, although it was reported that rainbow trout that ingested maslinic acid at the dose of 25 and 250 mg/kg for 225 days displayed an increase of the liver size of 52% and 40% with respect to the controls [22]. The treatment of mice with 50 mg/kg of maslinic acid, induced an increase on the relative weight of the brain of 11% ($P < 0.05$) higher than the control group, but changes in relative brain weight are rarely associated with neurotoxicity [25].

Overall, the results obtained in the toxicological study that showed an absence of harmful effects found in the hematology, clinical biochemistry, and histopathology evaluation suggest a large safety margin for this pentacyclic triterpene after oral administration.

4. Oral bioavailability of maslinic acid

The absence of adverse effects demonstrated for maslinic acid constituted a promising starting point for its future use as a nutraceutical due to the biological activities described for this pentacyclic triterpene. However, this information needed to be completed with a detailed study about its absorption, distribution, and metabolism either in humans or in animals after the consumption of this molecule. Bioactive compounds from food must be bioavailable in order to perform their beneficial effects on health. Therefore, bioavailability and metabolism of maslinic acid had to be explored *in vivo* in order to elucidate its mode of action and clarify the extent of its absorption and metabolism.

4.1. Plasmatic concentrations of maslinic acid

The bioavailability of maslinic acid was assessed after the p.o. administration of 50 mg/kg and the i.v. administration of 1 mg/kg to overnight fasted male Sprague-Dawley rats. Blood was taken from the lateral saphenous vein at different time points from 1 min to 24 h in order to cover the whole plasma concentration *versus* time curve [26]. Sampling was performed following a sparse design with three to six replicates for each time point. Blood was centrifuged and plasma was separated from

cells, and frozen at -20 ºC until analysis. The method to determine maslinic acid from plasma consisted of two extractions with ethyl acetate, prior to evaporation to dryness and reconstitution with methanol 80%. This pentacyclic triterpene was detected by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) [26].

The method to determine maslinic acid from plasma was validated according to the EMA Guidelines on Bioanalytical Method Validation [27]. The validation was performed with blank plasma samples spiked with maslinic acid at eight different concentrations (0.05-10 µM) and the parameters evaluated were recovery, matrix effect, linearity, sensitivity, precision, accuracy, selectivity, and carry-over [26].

The validated analytical method yielded excellent separation and detection of maslinic acid and the I.S. (betulinic acid) in plasma samples, as can be seen in Fig. 2.

The recovery of maslinic acid was calculated as the ratio between the mean peak area of extracted calibration standard samples and the mean peak area of the blank plasma samples spiked with this pentacyclic triterpene after extraction. Eight different calibration standards in the range 0.05 ± 10 µM were evaluated and the mean recovery was 99.0 ± 0.9% (n = 31). The sensitivity was evaluated on the basis of a signal to noise ratio of 3 for the LOD and 10 for the LOQ. The analytical method had an adequate sensitivity for the measurement of maslinic acid in plasma samples,

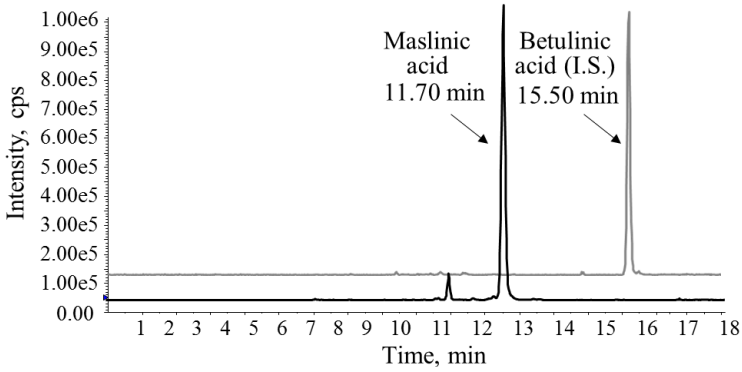

Figure 2. Representative HPLC-APCI-MS chromatogram obtained in negative mode with SIM acquisition at m/z 471.3 for maslinic acid and m/z at 455.5 for betulinic acid (I.S.). Rat plasma was obtained 45 min after the oral administration of 50 mg/kg of maslinic acid.

since the LOD was 2 nM and the LOQ was 5 nM. The LOD, verified by analyzing six blank plasma samples to which the theoretical concentration had been added, yield a concentration of 5.03 ± 0.13 nM with a precision of 6.22%. The linearity was assayed within the concentration range of 0.010-30 µM, which covered the expected concentration range for both the i.v. and p.o. administration routes. Linearity was observed in the calibration curves over the range 0.010-20 µM with a correlation coefficient up to 0.99, losing the linear tendency at 30 µM. The intra-day and inter-day precisions of the method were below 5.46% and 8.38%, respectively, in all the concentrations evaluated (0.05-20 µM). Finally, accuracy was established by comparing the nominal concentrations and the calculated concentrations. The deviation was lower than 4.82%.

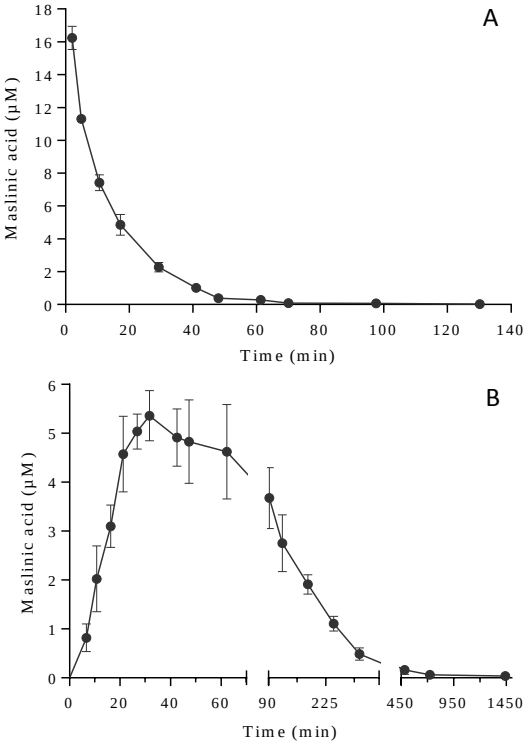

Figure 3. Plasmatic concentrations *versus* time profile of maslinic acid after single i.v. (1 mg/kg) (A) and p.o (50 mg/kg) (B) administration to overnight fasted rats.

The analytical method allowed the detection of maslinic acid after p.o. and i.v. administration, and the sampling times were adequate since in both cases the elimination phase was properly characterized. Fig. 3 shows the plasmatic concentrations *versus* time of maslinic acid after i.v. and p.o. administrations. After p.o. dosing maslinic acid was adequately quantified up to 24 h. Nevertheless, in the i.v. administration the blood collected at 8, 10, and 12 h yielded concentrations of maslinic acid below the LLOQ and were below the LOD at 24 h. Therefore, these concentrations were not included in the subsequent pharmacokinetic analysis [26].

4.2. Pharmacokinetic analysis of maslinic acid

The plasmatic concentrations *versus* time data were analyzed with the WinNonlin software to calculate the pharmacokinetic parameters. Non-compartimental and compartimental analyses were performed. Plasmatic data was best adjusted to a two-open compartments with first order absorption and linear elimination process (Fig. 4) [26].

Following p.o. administration, the peak plasma concentration after oral dosing (Cmax) was 4.82 µM and the time to peak plasma concentration (Tmax) was 30 min with an oral bioavailability of 6.25% [26]. The low oral bioavailability observed was in accordance with the results obtained for similar pentacyclic triterpenes [28,29]. A value of 2.3% was reported for 23-hydroxybetulinic acid after the p.o. administration of 200 mg/kg to mice [29]

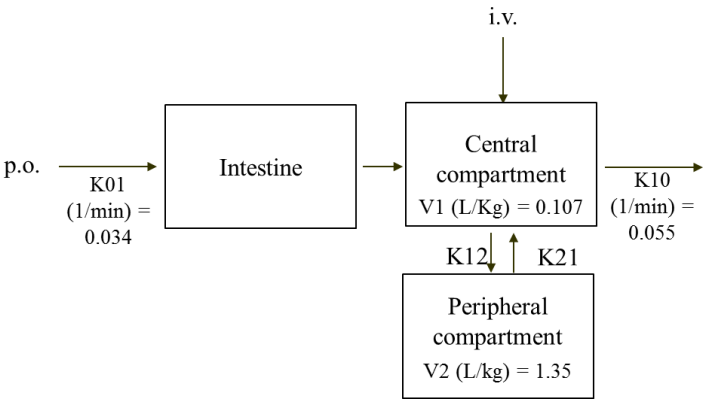

Figure 4. Schematic representation of the pharmacokinetic model that best describes the data of plasmatic concentrations of maslinic acid *versus* time after p.o. and i.v. administration.

and a 0.7% was indicated for oleanolic acid after the oral dosing of 25 and 50 mg/kg to mice [28]. The low oral bioavailability found for maslinic acid could be attributed to either a poor gastrointestinal absorption or a first-pass effect of the compound at the intestine or the liver. Maslinic acid have been described to exert a chemopreventive activity on colon cancer either *in vitro* in HT-29 colon cancer cells [30] or *in vivo* in Apc(Min/+) mice [31]. Then, if maslinic acid is poorly absorbed in the gut wall, then, the large intestine would face important quantities of this pentacyclic triterpene, thus becoming a target organ where maslinic acid could elicit the described protective activity against colon cancer.

The absorbed maslinic acid that reached the blood was distributed to the central (0.107 L/kg) and peripheral (1.35 L/kg) compartments. These results suggest an extensive distribution into tissues, since the total distribution volume (1.46 L/kg) is higher than the total body water in the rat (0.17 L/kg, for a mean weight of 0.28 kg). Similar distribution values were indicated for oleanolic acid (0.451 L/kg) after the i.v. injection of 1 mg/kg [28]. The estimated total plasma clearance (CL, 0.348 L/h/kg) was slightly lower than that of oleanolic acid (1.98 L/h/kg) [28]. The clearance value could be attributed to small hepatic and renal metabolism with unaltered renal excretion of this triterpene. Noteworthy, oleanolic, ursolic, and maslinic acids have been determined in several organs after a 4-week consumption of a diet with 0.5% of these triterpenes, and the liver had the largest concentrations followed by the kidney [32]. This results suggest that, liver could be an organ for maslinic acid storage, where this pentacyclic triterpene could exhibit a protective activity against hepatic diseases, as it has been demonstrated in different *in vitro* experiments [16,33].

5. Metabolism

The results obtained in the pharmacokinetic study showed a low oral bioavailability that could be attributed, in part, to metabolism. Therefore, the identification of the biotransformation of maslinic acid constitutes an important step in the investigation of this bioactive compound. In addition to the role that metabolism may have in affecting the bioavailability by influencing the distribution and the rate or the route of excretion, metabolites could either contribute to the biological activities or have toxicological effects independent of the parent compound [34]. Moreover, the identification of metabolites is important since they could act as

biomarkers of intake in dietary intervention studies [35]. With the aim of gaining insight into the bioavailability of maslinic acid, the metabolism was studied in rat plasma obtained after the oral administration of a dose of 50 mg/kg. Plasma samples were extracted with ethyl acetate prior to liquid-chromatography-atmospheric pressure ionization-linear trap quadrupole-ORBITRAP-mass spectrometry (LC-LTQ-ORBITRAP-MS) analysis [36]. This high-resolution mass spectrometer provides robust, accurate mass data that enables the unambiguous identification of metabolites. Plasma was screened for metabolites with the assumption that this bioactive compound from the diet could be considered as a xenobiotic by the organism and consequently, the detoxification processes that increased the hydrophilicity of maslinic acid could be activated [37]. Hence, metabolites arising from phase I reactions, which are based on oxidation, reduction, and hydrolysis processes, and phase II biotransformations, such as glucoronidation, sulfation, methylation, acetylation, and glutathione conjugation were searched in the samples. The results obtained showed that only compounds arising from phase I reactions were found. The molecule of maslinic acid was modified in order to yield four monohydroxylated derivatives (M1a-M1d), one monohydroxylated and dehydrogenated metabolite (M2) as well as two dihydroxylated and dehydrogenated compounds (M3a-M3b) [37].

Metabolism of maslinic acid has only been investigated *in vitro* with the use of fungi such as *Cunninghamella* [38] and *Rhizomucor* [39,40]. These microorganisms are commonly used in the evaluation of the metabolism of drugs and xenobiotics since they express cytochrome P450 (CYP) enzymes that allow equivalent phase I reactions to mammalian metabolism. In these studies, the chemical structure of the derivatives was elucidated based on the use of nuclear magnetic resonance and high-resolution mass spectra analyses. Therefore, the hydroxyl of the mono-hydroxytaled metabolite (M1-a, M1-b, M1-c, M1-d) could be compatible with the metabolites previously described [38,39,40]. The transformations found after incubations with *Cunninghamella blakesleana* were three different monohydroxylated derivatives (7β-hydroxy, 15α-hydroxy, 13β-hydroxy with double bound migration) [38]. When *Rhizomucor miehei* was used, monohydroxylation was also observed, but in this case the addition of the hydroxyl group took place at the angular methyl group on position 30 [39].

Moreover, the monohydroxylated and dehydrogenated metabolite (M2) could be formed by a hydroxylation at C-11, followed by a

dehydrogenation to ketone or by the formation of an epoxy group between C-11 and C-12, as previously suggested [39,40]. The dihydroxylated and dehydrogenated metabolite (M3-a, M3-b) could be compatible with the hydroxylation at C-30 and the epoxy between C-11 and C-12 [39]. However, the two observed metabolites could also be due to other structures not detected in the incubations of maslinic acid with fungi [38-40] such as the combinations of these functionalizations or the presence of a hydroxyl group in one position and a ketone in another carbon.

The only *in vivo* studies were performed with pentacyclic triterpenes similar to maslinic acid and confirmed that the biotransformation of these compounds occurs mainly by phase I reactions [41,42]. The oral administration of 12.5 mg/kg of boswellic acids to female albino Wistar rats gave in plasma two monohydroxylated derivatives [56]. Incubation of oleanolic acid [28] and boswellic acids [41,42] with rat and human liver microsomes also indicated that hydroxylation is the main metabolic route for pentacyclic triterpenes.

The quantification by LC-APCI-MS showed that maslinic acid was the most abundant compound in plasma (81.8%), which indicates that metabolism is low. It was followed by metabolite M2 with a 13.2% and M1-a with a 2.5%. The other metabolites (M1-b, M1-c, M1-d, M3-a, M3-b) were found in minor amounts since all of them accounted for only a 2.5% [37]. Overall, the study gives a comprehensive insight into the metabolite profile of maslinic acid, thus increasing our knowledge about the bioavailability of this bioactive food component.

6. Conclusion

Maslinic acid is a bioactive compound that targets a wide variety of molecules or metabolic pathways suggesting its putative use as nutraceutical. However, research on maslinic acid is currently in the early stages of the pre-clinical phase. Consequently, the present chapter summarizes the current knowledge about the safety and the bioavailability of maslinic acid after oral administration. A liquid-liquid extraction method followed by LC-APCI-MS was validated to determine of maslinic acid in blood. Analysis of plasmatic concentrations indicated that this bioactive compound was absorbed with a peak plasmatic concentration at 30 min and oral bioavailability of 6.25%. Metabolism was subsequently screened and revealed that maslinic acid is the main compound in plasma although it undergoes mainly phase I metabolism through hydroxylation

and oxidation reactions, leading to seven metabolites. In summary, the present results show that maslinic acid can be safely administered by the oral route and provides a comprehensive knowledge on the bioavailability and metabolism as a first step on the use of this bioactive compound as a nutraceutical.

Acknowledgements

This work was supported by grants AGL2009-12866 and AGL2013-41188 from the Ministerio de Economía y Competitividad and 2009SGR471 and 2014SGR1221 from the Generalitat de Catalunya, Spain.

References

1. Espín, J. C., García-Conesa, M. T., Tomás-Barberán, F. A. 2007, *Phytochemistry*, 68, 2986.
2. Lozano-Mena, G., Sánchez-González, M., Juan, M. E., Planas, J. M. 2014, *Molecules*, 19, 11538.
3. Bächler, L. 1927 'Monographie der Mehlbeeren'; Impr. et Édition des Dernières Nouvelles: Colmar, France.
4. Caglioti, L., Cainelli, G., Minutilli, F. 1961, *Chim. Ind.*, 43, 278.
5. Vioque, A., Morris, L. 1961, *J. Am. Oil Chem. Soc.*, 38, 458.
6. Bianchi, G., Pozzi, N., Vlahov, G. 1994, *Phytochemistry*, 37, 205.
7. Stiti, N., Triki, S., Hartmann, M. A. 2007, *Lipids*, 42, 55.
8. Kombargi, W. S., Michelakis, S. E., Petrakis, C. A. 1998, *J. Econ. Entomol.* 91, 993.
9. Caligiani, A., Malavasi, G., Palla, G., Marseglia, A., Tognolini, M., Bruni, R. 2013, *Food Chem.*, 136, 735.
10. Banno, N., Akihisa, T., Tokuda, H., Yasukawa, K., Taguchi, Y., Akazawa, H., Ukiya, M., Kimura, Y., Suzuki, T., Nishino, H. 2005, *Biol. Pharm. Bull.*, 28, 1995.
11. Xu, H. X., Zeng, F. Q., Wan, M., Sim, K. Y. 1996, *J. Nat. Prod.*, 59, 643.
12. Yoshida, T., Okuda, T., Memon, M. U., Shingu, T. 1985, *J. Chem. Soc., Perkin Trans.*, 1, 315.
13. Romero, C., García, A., Medina, E., Ruiz-Méndez, M. V., de Castro, A., Brenes, M. 2010, *Food Chem.*, 118, 670.
14. Pérez-Camino, M. C., Cert, A. 1999, *J. Agric. Food Chem.*, 47, 1558.
15. Kalogeropoulos, N., Chiou, A., Ioannou, M., Karathanos, V. T., Hassapidou, M., Andrikopoulos, N. K. 2010, *Food Chem.*, 121, 682.
16. Lin, C. C., Huang, C. Y., Mong, M. C., Chan, C. Y., Yin, M. C. 2011, *J. Agric. Food Chem.*, 59, 755.

17. Li, G. L., You, J. M., Song, C. H., Xia, L., Zheng, J., Suo, Y. R. 2011, *J. Agric. Food Chem*., 59, 2972.
18. Organisation for Economic Cooperation and Development. Guidelines for testing chemicals. Repeated dose 28-d oral toxicity study in rodents, no. 407, 2008. OECD, Paris, France.
19. Sánchez-González, M., Lozano-Mena, G., Juan, M. E., García-Granados, A., Planas, J. M. 2013, *Mol. Nutr. Food Res.*, 57, 339.
20. Rufino-Palomares, E., Reyes-Zurita, F. J,, Fuentes-Almagro, C. A., de la Higuera, M., Lupiañez, J. A., Peragón, J. 2011, *Proteomics*, 11, 3312.
21. Hidalgo, M. C., Skalli, A., Abellén, E., Arizcun, M., Cardenete, G., 2006, *Aquacult. Nutr*., 12, 256.
22. Fernández-Navarro, M., Peragón, J., Amores, V., De La Higuera, M., Lupiáñez, J. A., 2008, *Comp. Biochem. Physiol. C Toxicol. Pharmacol.*, 147, 158.
23. Liu, J., Sun, H., Duan, W., Mu, D., Zhang, L., 2007, *Biol. Pharm. Bull*., 30, 2075.
24. Tang, X. Z., Guan, T., Qian, Y. S., Li, Y. M., Sun, H, B., Huang, J. H., Zhang, Y. 2008, *Chin. J. Nat. Med.*, 6, 53.
25. Sellers, R. S., Morton, D., Michael, B., Roome, N., Johnson, J. K., Yano, B.L., Perry, R., Schafer, K. 2007, *Toxicol. Pathol*., 35, 751.
26. Sánchez-González, M., Colom, H., Lozano-Mena, G., Juan, M. E., Planas, J. M. 2014, *Mol. Nutr. Food Res.*, 58, 1970.
27. European Medicines Agency (EMEA), Committee for Medicinal Products for Human Use (CHMP). 2011, Guideline on Bioanalytical Method Validation, London.
28. Jeong, D. W., Kim, Y. H., Kim, H. H., Ji, H. Y., Yoo, S. D., Choi, W. R., Lee, S. M., Han, C. K., Lee, H. S. 2007, *Biopharm. Drug Dispos.*, 28, 51.
29. Yang, M., Wang, G. J., Wang, S. J., Li, X. T., Xu, Y. P., Wang, S. P. Xiang, J. D. Pan, S. R. Cao, G. X., Ye, W.C. 2005, *Rapid Commun. Mass Spectrom.*, 19, 1619.
30. Juan, M. E., Planas, J. M., Ruiz-Gutiérrez, V., Daniel, H., Wenzel, U. 2008, *Br. J. Nutr*., 100, 36.
31. Sánchez-Tena, S., Reyes-Zurita, F.J., Díaz-Moralli, S., Vinardell, M. P., Reed, M., García-García, F., Dopazo, J., Lupiañez, J. A., Günther, U., Cascante, M. 2013, *PloS One*, 8: e59392.
32. Yin, M. C., Lin, M. C., Mong, M. C., Lin, C. Y. 2012, *J. Agric. Food Chem.*, 60, 7697.
33. He, X., Liu, R. H. 2007, *J. Agric. Food Chem.*, 55, 4366.
34. Holcapek, M., Kolárová, L., Nobilis, M. 2008, *Anal. Bioanal. Chem.*, 391, 59.
35. García-Cañas, V., Simó, C., León, C., Cifuentes, A. 2010, *J. Pharm. Biomed. Anal.*, 51, 290.
36. Benedetti, M. S., Whomsley, R., Poggesi, I., Cawello, W., Mathy, F. X., Delporte, M. L., Papeleu, P., Watelet, J. B. 2009, *Metab. Rev.*, 41, 344.
37. Sánchez-González, M., Lozano-Mena, G., Parra, A., Juan, M. E., Planas, J. M., 2015, *J. Agric. Food Chem.*, 63, 1126.

38. Feng, X., Luan, J., Guo, F. F., Li, D. P., Chu, Z. Y. 2012, *J. Mol. Catal. B: Enzym.*, 82, 127.
39. Martinez, A., Rivas, F., Perojil, A., Parra, A., Garcia-Granados, A., Fernandez-Vivas, A. 2013, *Phytochemistry*, 94, 229.
40. Martínez, A., Perojil, A., Rivas, F., Parra, A., Garcia-Granados, A., Fernandez-Vivas, A. 2015, *Phytochemistry*, 117, 500.
41. Krüger, P., Daneshfar, R., Eckert, G. P., Klein, J., Volmer, D. A., Bahr, U., Müller, W. E., Karas, M., Schubert-Zsilavecz, M., Abdel-Tawab, M. 2008, *Drug Metab. Dispos*., 36, 1135.
42. Gerbeth, K., Hüsch, J., Fricker, G., Werz, O., Schubert-Zsilavecz, M., Abdel-Tawab, M. 2013, *Fitoterapia*, 84, 99.