Comprehensive extraction and NMR-based Metabolomics : novel approaches to natural products lead finding in drug discovery

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Summary

Obesity has become a serious global health problem both in developed and developing countries. It is also an extremely expensive health problem since it associates with several diseases such as cardiovascular diseases, type 2 diabetes, degenerative joint diseases and cancer, moreover it diminishes the quality of life of affected individuals. Currently, Orlistat is the only approved obesity drug being used worldwide. On the other hand, commercial non-prescription dietary supplements for weight loss management are available abundantly in the market and among them supplements originating from plants are predominant. Scientific studies on anti-obesity activity of medicinal plants are also abundant. Many plants have been studied for different mechanisms of anti-obeisty activity, and the respected active compounds have beed identified. However, the elaborate studies are not counterbalanced with a significant number of compounds/extracts in clinical trials. Besides lack of safety data in humans, the major problem is to define a reasonable effective dose (**Chapter 1**).

The discovery of new drugs from plant sources may involve several approaches, such as random screening, follow up reported biological activities, or an ethnopharmacological approach. The last two were used to screen medicinal plants and spices for several anti-obesity related activities (adenosine A1 receptor, CB1 receptor, TNF-α inhibition, and lipolytic activity on 3T3-L1 adipocyte). Several spices and medicinal plants were found to be highly active in one or more bioassays tested. The tested concentration varied depending on the bioassay but all were less than 100 μM. Sesame seeds, red chili, and *B. hispida* seed show high binding activity to the adenosine A1 receptor; nutmeg, mace, black pepper, and turmeric have high binding activity to the cannabinoid CB1 receptor; mulberry stem bark, temulawak, and temukunci have high binding activity for both receptors; piment and turmeric showed high inhibition of TNF-α accumulation, while black onion seeds are the only spice having high activity for induction of lipolysis in 3T3-L1 adipocyte. Several major compounds found in spices (capsaicin, myristicin, papaverine, piperine, cinnamic acid, α-pinene, camphene, and borneol) were tested in the respective bioassays but they did not show any activity. The

activity was probably the result from synergistic effects among different compounds (**Chapter 2**).

Orthosiphon stamineus is a popular medicinal herb in Indonesia. It is used to treat various diseases such as hypertension, diabetes, and kidney stone. It is also used as an ingredient in diuretics and herbal slimming preparations. Many compounds have been identified in this plant including diterpenoids, triterpenoids, and flavonoids (297, 298, 313-315). We chose this plant to find new ligands for adenosine A1 receptor by using conventional bioassay guided fractionation, which resulted in the isolation of 7 methoxy- flavonoids with Ki values in the micromolar range. The Hill slope values are not significantly different from unity (0.9 – 1.4), indicating an antagonistic effect to the receptor. The results thus provide evidence for the traditional use of *O. stamineus* as slimming herbal or kidney stones (**Chapter 3**).

Rapid developments in the modern drug discovery process, such as combinatorial chemistry and structure activity relationships, neglected natural products, and particularly plants, as a source of novel leads. Despite the rich diversity in molecular structures and biological functionality, the complex matrix of the compounds with a broad dynamic range has limited the use of plants as an important source for drug development. Many new strategies to overcome these bottlenecks have been reported, such as constructing a better natural product library, improving fractionation methods, improving dereplication and identification steps, and applying natural product virtual screening. However, no significant improvement was achieved. Actually, the most needed innovation in drug prospecting from plants is the rapid dereplication of known active compounds, false positive compounds, and identification of novel active compounds. Adressing this issue, several papers have described the use of the more holistic approach targeting on a wide range of metabolite present in plant extract, i.e. metabolomics. This new approach involves the use of various analytical methods followed by appropriate multivariate data analysis. The aim is to shorten the bioassay guided isolation route, particularly in the identification and dereplication step (**Chapter 4**).

Thin layer chromatography was combined with orthogonal-partial least square (OPLS) analysis to correlate chemical profile of *O. stamineus* extract obtained from liquid-liquid partition with its adenosine A1 receptor binding activity. The degree of

correlation was observed by comparing the color intensity of the spot in the loading plot, in which spots 1-5, and 7 were considered to be active. The retention time of seven methoxy- flavonoids previously identified as active to the receptor coincided with spots 2, 3, 4, and 5. Pilloin and eupatorin were identified in spot 2, 3'-hydroxy-4',5,6,7-tetramethoxyflavone and sinensetin in spot 3, tetramethylscutellarein and eupatoretin in spot 4, while 5,6-dihydroxy-7,4'-dimethoxyflavone in spot 5. Further confirmation was performed by applying ^{1}H NMR profile of the extracts to OPLS analysis. The results coincided with the pattern obtained with TLC (**Chapter 5**).

A novel extraction method, namely comprehensive extraction, was developed. In this approach plant material is continuously extracted with a gradient of solvents of increasing polarity, resulting in partly overlapping fractions. Based on the results of hierarchical cluster analysis (HCA) and partial least square-discriminant analysis (PLS-DA), comprehensive extraction was found to be reproducible, faster and efficient to provide more clear ^{1}H NMR spectra of the total chemical profile of plant materials (**Chapter 6**).

Comprehensive exraction and NMR metabolomics coupled to multivariate data analysis (partial least square and orthogonal-partial least square analysis) were used to identify bioactivity-related metabolites from crude plant extracts. Although both statistical methods are statistically valid, the orthogonal-partial least square analysis (OPLS) was found to be the most suitable tool to study the correlation between chemical profiles of plant extracts with their bioactivity profile. It is easier to interprete than partial least square (PLS), since the variables uncorrelated with the response are orthogonalized from the correlated ones. The broader polarity solvents combination (*n*-hexane-acetone-water) gives a better chemical variation than the narrow one (ethyl acetate-methanol (1:1)/methanol-water (1:1)). Chemical validation was performed by cross-checking the ^{1}H NMR, J-res, COSY, and HMBC NMR data from seven flavonoids previously reported to bind to the adenosine A1 receptor with one of the active fractions obtained from comprehensive extraction. All important signals of these active flavonoids have positive Y-related coefficient values and a value of importance (VIP) values higher than 0.5, meaning that these compounds significantly contribute to the high avtivity of this fraction (**Chapter 7**).

Various compounds have been isolated from different parts of *Morus alba* L. (fruits, leaves, root bark, and stem bark) (303, 428, 429, 437-444). Prenylated aromatic compounds and alkaloids were found to be the most abundant. The methanolic extract of this plant has a high binding activity to the adenosine A1 receptor (**Chapter 2**). Comprehensive extraction, NMR metabolomics and OPLS were applied to estimate which compounds correlate significantly with the activity. Fatty acids signals were found to have a strong poisitive correlation to the activity of the extracts, but not in the case of alkaloids and prenylated compounds signals. Characteristic signals of two compounds previously isolated from *M. alba* also have negative Y-related coefficients. This is in accordance with the results of the bioactivity test in which these two compounds did not show any binding activity to the receptor (**Chapter 8**).

This thesis describes a new strategy to improve dereplication and identification steps in drug discovery process from plants. The integration of comprehensive extraction coupled to NMR metabolomics and multivariate data analysis is found to be a potential new approach to uncovering bioactive compounds from crude plant extracts.