Protocol: Extract bioactive substances from plants – a practical scientific guide

***Be aware**: The beauty of plants and their variety of bioactive compounds poses also a serious threat. A lot of plants contain toxic compounds. Even with low concentration of compounds occurring in the original plant material, be aware of "sola dosis facit venenum" – the dose makes the poison. Sometimes an extract or even the working process can be extremly dangerous due to the high concentration of substance. For example **NEVER** try to extract nicotine from tobacco plants or cigarettes. Nicotine is highly toxic and can be absorbed easily by the skin. If you are not sure what plant you are dealing with be careful or in question don´t do it. Furthermore handle chemicals as solvents careful due to it health related concerns and especially to its flammability. **Never** work with flames or hot plates near to organic solvents. All experiments are performed under your own risk and responsibility. Always: Safety first.*

Background

What are we looking for to extract from plants?
In general natural products are all chemical substances produced by living organisms. But if you are looking for bioactive compounds a further differentiation is also good to know: primary and secondary metabolites. Everything what is directly linked to the normal growth, development, or reproduction of an organism is a primary metabolite. If a compound isn´t linked to immediate survival, but gives the organism a long-term "competitive advantage" like survivability, fecundity, or even aesthetics it is a secondary metabolite. For example caffeine is produced by the plant not to give us a quick start each morning, but to defend itself with a bitter taste which even can act neurotoxin against other organism which try to eat the beans or leaves. So with producing caffeine the plant increases its chances to propagation. Everything comes done to mating or killing in nature.

What makes extractions so difficult?
Our goal is to get a certain substance from plants and get ride off all the rest like the plant tissue. Please keep in mind, even with some simple methods helping you to physically or chemically separate most of it, you still might have a complex chemical mixture of similar compounds: crude extracts. And depending on how precise your extraction is supposed to be. Isolating one particular compound can vary from easy to hard and even impossible in some rare cases. Even skilled chemist sometimes need for certain natural products high end equipment to get the job done. Another issue to keep in mind is that sometimes need a lot of plant material to even obtain little of your desired product. Nevertheless, don´t be intimidated, because with a lot of basic extractions you can get pretty far and it get most of the jobs done. Proof of concept is that a lot of plant extracts are used for medical purposes since ages.

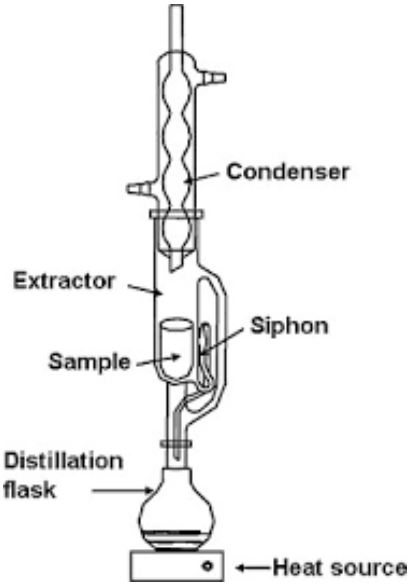
Principles

What are the basic steps in extraction?

There are 5 basic steps working on plant extraction, but there can be plenty iterative cycles of

1) Size reduction
2) Extraction
3) Filtration
4) Concentration
5) Further isolation and purification (optional)

Let´s get into some details for each step to get a better understanding of the general process:



Picture 1: Coffee machine (left), Soxhlet-Apparatus (right) = Extraction of secondary metabolites in daily life and in chemistry lab. Everyone knows how to brew a coffee, but how does a Soxhlet-apparatus works in the lab. It works quite similar, you heat a solvent which is in the flask at the bottom. The solvent evaporate and similar to a distillation it will condensate because of the water cooling in the top. From there it will drop down onto the sample. The siphon allows the solvent to get back to the solvent flask but also guarantee the right amount of solvent to have a steady extraction. The solvent will be extract chemicals from the sample in a steady way. Imagine a coffee machine in which the water of the coffee will be re-used to extract coffee over and over again. Though similar – never use kitchen equipment with chemicals.

First of all you need to size down the plant material by picking the most promising part of the plan cutting it, crushing it and/or grinding it. This not only matters because of practical reasons, but also to rupture the plant and cell tissue and make ingredients exposed to the extraction. Furthermore physically speaking it increase the surface area which also enhance the extraction performance. But no need to push it to hard.

The extraction mostly is based on a physically transfer from the original plant material into another phase which can be separated by filtration. This can be a liquid or even gas depending on the physical properties of the desired compound and the used method. Most common is for the start to use a mixture of organic solvent as ethanol or acetone. Some target molecule might not be soluble in organic solvents so use water instead. Lab experience sometimes show that changes in pH or the temperature get a better result. This is based on the fact that solubility is depend on this kind of factors. If applying heat first of all be careful because of your organic solvents (flammable and explosive!), also be aware heat might destroy or vaporize some compounds. How much of the solvent should you use? As a rule of thumb use enough to cover the plant material sufficiently, but adding to much just makes your live harder later on. Preferably repeat the extraction after the filtration, some methods already apply this repeated extraction in their setup of apparatus (Soxhlet). In some cases you get decent result with none additional solvent for example to extract dyes from plants in its own cellular water by pressure and filtrating it.

Filtration is most of the time straightforward. But – especially if you not plan to do a repeated extraction of the left over plant material - it might help to “flush” with some fresh solvent, because sometimes crystallization already starts in the filter and hampers your yield.

Concentration of the extract means get ride of the solvent. You need to evaporate the organic solvent. This can be achieved either passively by just let it dry openly over night or days. Or in case of ethanol to carefully boil it off. In labs you normally use rotary evaporation, but this equipment is expensive and can be dangerous if handled incorrectly. The more solvent you used the more time consuming this step will be, this is why you shouldn´t use to much in the first place.

The result of a simple ethanol extraction is most of the time a brown-ish, colour-ish sticky fluid or oil as raw extract. Sometimes you might get already crystals in this raw extract, which brings us to a very common method for basic purification. It is called re-crystallization and takes advantage of the different solubilities of chemical compounds in different temperatures. The idea is adding as little of warm/hot solvent as needed to resolve all the raw extract. Add the solvent drop by drop, shake it and apply carefully heat for example in a sealed test tube with a warm water bath for example (flammability and expansion of solvents in gas phase). If everything is resolved, let the solution cool down or even put it in the fridge (one just for chemicals). Some compounds might crystalize because they have a worse solubility in cold temperatures, so you are able to filtrate and separate them from the rest. A basic advice is that as long as you don´t know in which phase your desired substance is solved, never discharge anything.

Example –Extraction of Cannabinoids and other metabolites from *Cannabis sativa*

Background:
Search for antimicrobial substances in the BioStrike Project at the Waag Society. First plant of interest tulip as a "typical" dutch flower. After a walk through the canals in Amsterdam had the idea to check if Cannabis as medical plant is also suitable as antibiotic. Literature research revealed this results:
(1) G. Appendino *et. al* "Antibacterial Cannabinoids from Cannabis satiWa: A Structure-Activity Study" *J. Nat. Prod.* **2008,** *71,* 1427–1430.

(2) L. L. Romano, A. Hazekamp „Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine" *Cannabinoids* **2013**;1(1):1-11

(3) N. Schultz "A New MRSA Defense" - www.technologyreview.com/s/410815/a-new-mrsa-defense/

Especially (2) shows a nice comparison of different extraction protocols. Due to the DIY approach and the use of harmless substances the extraction with olive oil was chosen. The cannabis plant material was bought at a local coffeeshop in Amsterdam and it was 1 gr.

1) Cut plant material, grind it to small pieces and put it in a sealable 50 ml tube
2) Literature used 100 ml for 10 gr, so tried 10 ml for 1 gr, but wasn´t covering the plant material →add 20 ml of olive oil to plant material
3) Put sealed tube into boling water bath for 1-2 h
4) Cool down the sample
5) Filtrate it with simple coffee filter

Result: oil, raw extract with characteristic smell of cannabis

Further experiments: Test oil extract prepared on medical paper for antimicrobial activity in a petri dish against several strains, showed positive results e.g. against *bacillus subtilis*

Examples for poison Plants – DO NOT TOUCH:

Do not pick plants from botanical garden! They look nice, but most of them are very toxic. Even common plants can be toxic. So be careful and **NEVER** perform random experiments without knowing what you are dealing with.

Picture	Name	Poison / toxicity
	*Aconitum napellus* (monk's-hood)	Aconitin / high +++
	*Colchicum autumnale* (autumn crocus, meadow saffron)	Colchicin / high ++
	*Convallaria majalis (*Lily of the valley)	k-Strophanthidin / high ++
	*Solanum lycopersicum (tomato)*	Solanin / high + especially high concentration in raw = green tomatos, same toxin is raw potatoes
	*Hedera helix* (common ivy)	Saponine / +