PHARMACOGNOSY AND PHYTOCHEMISTRY

Dr. Galvina Pereira

Department Of Pharmaceutical Science and Technology

Institute Of Chemical Technology Mumbai

Week 11

Lecture 55

Quantitative Evaluation of Herbal Drugs Using Biological Methods

Hello everyone, and welcome back to week 11 of the NPTEL course in Pharmacognosy and Phytochemistry. This week, we are again exploring different methods of quality control. In this session, we will examine a slightly different method of quality control: the biological method. Before we proceed to the biological method, let's do a quick recap. So far, we have covered organoleptic, microscopic, physical, and chemical methods of analysis.

All these methods help us ascertain the quality—identity, purity, and content—of the herbal drug material. Now, what are these biological methods? When we say biological methods, we refer to biology. This means we will use living organisms, either as whole organisms, enzymes, products, or certain cells purified from them.

In other words, we will perform a method of analysis. This quality control method involves using biological specimens, samples, enzymes, or, in certain cases, fluids. Let's explore the different biological methods used for herbal quality control. Most biological methods we discuss are quantitative.

In this session, we will cover methods such as the hemolysis test, bitterness value, microbial contamination, and efficacy studies. Now, efficacy studies is a large domain. So efficacy studies means when I'm consuming a drug, is it really going to be showing its activity as promised? Now, this can be done by actually administering the drug.

to certain individuals or in some cases to the lab animals or experimental animals more specifically or in some cases it can be observed by expression or suppression of certain

proteins and enzymes in cells which are grown ex vivo So efficacy studies is going to tell you whether your medicine is going to work or not. It's a big and a very broad area and efficacy studies varies as per the therapeutic value intended. So if I have to study for anti-diabetic effect, I'll have different kind of assays.

If I have to do anti-cancer effect, I'll have a different kind of assays. So efficacy studies being a little bigger domain, we will not be covering in this course. But in today's session, we'll be covering more specifically the hemolysis test, bitterness value and how do you assess microbial contamination in given sample. So let's start with the first sample and that's your hemolysis test.

Now hemolysis as the name indicates somewhere you are going to take this blood and you are going to hemolyze it. That means you are going to cause the lysis of your RBCs. Now why does this happen? So there are certain compounds in plants that are called as saponins. Now saponins have a good ability to play with permeability of your RBC membrane

thereafter disturbing it and kind of eventually rupturing it. This happens by binding to different locations and the eventual product is the heme which is trapped inside the RBC is released. Now when this heme is released, your plasma becomes colored because of the presence of heme and that color you are going to see.

For that we will require few things and what we require in terms of biological sample is going to be your RBC suspension. Now when we say hemolysis you want to release the hemoglobin out of your RBC. So you are going to require RBC. So from where do you procure this RBC? This RBC can be procured from rabbit.

In some cases even mice are used but not clearly for this specific method. Bovine RBCs in some cases even human RBCs if at all they are available. In order to procure this RBCs, you procure blood first. So you draw blood out of animals, bovine animals, humans and this blood you are going to separate its component. You are going to separate the plasma, you are going to separate the WBC, RBC and so on.

This is done by centrifugating the blood at a very high speed or at a very high RPM wherein your cells settle based on their density. So you will see a centrifuge tube and in this centrifuge tube after centrifugation the top layer will be a very clear plasma. After plasma you will see the little less denser WBC and platelets. And because of the heme the bottom most plug or that bottom most layer is actually the RBC.

So you have different different cells settling at different different rate due to change in their weight and the bottom most layer is your RBC. So this what is done is this upper layers are carefully removed off and this RBC plug what is there is resuspended. Preferably in an anticoagulant initially till the plasma is there and later on using your phosphate buffer. Basically a saline phosphate buffer because isotonicity here is very important.

If you don't keep your solutions isotonicity because they will become . You know, if the solution becomes hypertonic or hypotonic, they might shrink or rupture. We don't want the cells or especially our RBC cells to rupture or shrink. And as a result, we keep it in a isotonic saline buffer solution suspended. So generally what you prepare in a lab is

somewhere between 1 to 5% RBC suspension in a solution of your saline phosphate buffer. Now you take this what you will see is again if you re-centrifuge it again your RBCs will settle. Now this time what you are going to do is you are going to add your RBCs to your test tubes containing different different solution. Now you cannot keep a really big test tube so imagine a very small test tube

and in each of it we have just added 0.1 ml of your RBC suspension. Now in addition to that you will add your extract. So in this case, say for example, I add here 0.1 ml extract, 0.2 ml extract, 0.3 ml extract, and 0.4 ml extract. Now, if at all it contains saponins, what is going to happen is, as the concentration increases, it is going to cause lysis of a large number of RBCs.

So, the more the amount of saponins, the more the lysis will happen. And what is the product of lysis? The product of lysis is heme, which is going to escape into this saline buffer. So initially, you will see very clear or very little hemolysis. But as the concentration of saponin increases, it becomes increasingly red.

That means more and more of this heme is released into the saline phosphate buffer. Now, what you do is, in order to quantify this, you can use colorimetry. So, just measure the optical density of this solution at about 630 nanometers and then use a standard saponin of which you know what is the LD50 or especially the HD hemolysis 50 value. Now, the HD50 value is the value of the standard saponin that causes 50% of the RBCs to lyse.

Now you will see that, and whenever your value matches that, you can say that this is the concentration which is the HD50. Now, you compare it versus the standard saponin; you already know the concentration of the standard saponin. So this is the concentration of standard saponin, say 100 micrograms per ml, which causes hemolysis of 50% of your drug. Now check the extract say your extract will also contain certain things so say for example you will say about

400 microliter causes lysis of about say I am just putting it 400 microgram per ml causes HD50. Now what does this mean? This means that you require 4 times more concentration of your test sample as compared to standard sample. So if this has 1 mg. So this has 0.25 mg of your saponin.
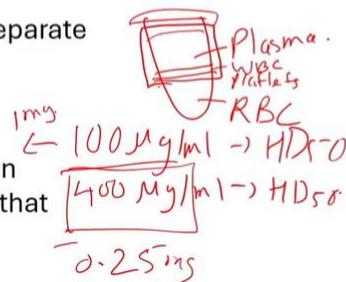
So this is how this method is used for quantification of saponin. Now we go to the next method. The next method is a little obsolete now because of machine learning and

electronic tongue along with HPLC. But this is a method which was earlier used and that is your bitterness value. Now what is bitterness value?

Bitterness value is the amount of bitterness that is felt after you consume Say, for example, dissolving one gram of quinine in two liters of water. So you take one gram of quinine, dissolve it in two liters of water, especially the salt quinine hydrochloride is used. And then when you see it effectively, this is the concentration that you reach 0.5 mg per ml. Now, that is considered to be a bitterness value of about 2 lakh units.

Now, compared to that, what dilution of your sample? Say, for example, there are numerous bitters, strychnine, brucine or even imitin, even your andrographolide or even we saw your iridoid, swertiamarin. So, you are going to take this solution and you are going to taste in a similar manner. So, you know, you're going to dilute it till you reach a threshold bitterness value and then you check what is going to be as equal to the same dilution or same bitterness as that of quinine diluted in 2 liters. Now, this can be given by a formula. 2000 by A, why 2000 by A is kind of reciprocal, 1 gram of quinine in 2000 liters, so how much is your sample, so A is actually the bitterness value is actually the reciprocal of the concentration, so you need to take it ulta and that is Exactly the reciprocal value of the concentration needed to match the bitterness of 1 gram of your quinine.

Now once this is done you put your A value and then you will get your bitterness value of the sample. Now, moving on to the next one, but a very important one, and that is determination of microbial contamination. Now, when you see your synthetic APIs, most of them have been synthesized by using chemical methods and as a result, They are not food for the microorganisms. Many of the chemicals don't really harbor microbial contamination.

But when you come to herbal drugs, they are all natural. They are carbohydrate rich. They retain certain amount of moisture. And as a result, they are more and more susceptible to microbial contamination. In that case, it is very important to

check the microbial contamination values, especially for your herbal drugs. Now, one important difference is that microbial contamination is essentially different from sterility.

Sterility refers to a term when you say there is a complete absence of microorganisms, whereas determination of microbial contamination Sterility is something where you say it's a complete lack or absence of

microorganisms, whereas here you will say that you're going to quantify how many microorganisms are there. The reason for this is most of the herbal drugs we consume via the oral route. And as a result, we have our stomach acids and our immunity, which will support our defense function. So as a result, these drugs or these herbs need not be sterile. But in some cases, if the microbial load is too much, imagine you trying to eat up the fungus.

What is going to happen is, somehow or the other, you are bound to get an infection. So there is a particular limit beyond which the microbial contamination should not be present. Your product need not be sterile, but your microbial contamination should be curtailed—that is the proper word. And that is why we do the determination of microbial contamination. Now, determination of microbial contamination has two important aspects.

First, we are going to see how many alive or viable microorganisms are surviving in it. And that is referred to as your TVAC or often called your total viable aerobic count. The reason we do a total viable aerobic count is that most of the herbs are kept exposed to the atmosphere. As a result, the majority of the microorganisms that survive on these herbs are aerobic. But yes, there is a chance that certain anaerobic microorganisms also survive, but

they are not obligatory anaerobes. So, the total viable aerobic count will give you a rough estimate of how much contamination your drug carries. Now, how are you going to do or measure the microbes in the herb? Now, in order to do this, you require something called a media. Now, this media will essentially help you in the growth of microorganisms as well as the enumeration of microorganisms.

Now, the growth of microorganisms and enumeration of microorganisms can be done in certain nutrient-rich media, often referred to as your nutrient broth. So, this nutrient broth kind of becomes home to those microorganisms. They multiply in that and give rise to

colonies. These colonies can then be counted in the case of a plate method, or this growth can be counted as an increase in turbidity.

The more microorganisms, the more difficult it will be for light to pass through. The solution will lose its clarity. Imagine fungus growing in water. So what is going to happen? Initially it's clear, now it is not.

So there is a change in optical density and that is also one way to do it. Now, we come to the second part. The second part is pathogenic microorganisms. Now, these are the microorganisms which definitely are going to be injurious to us if we consume it, especially if the patients consume it, already their health is compromised. And in that case, these microorganisms, especially the opportunistic ones,

What will they do is they will infect the patient and give rise to secondary disease. Say for example salmonella typhi can infect and then you will get typhoid in addition to the disease you are already suffering from. So we check this opportunistic but pathogenic microorganisms. Using again plate method as well as nowadays we have certain methods such as your polymerase chain reaction with ELISA which helps you detect this microorganisms even with a very lesser concentration.

So let us see this method what is total viable aerobic count. So in this case we generally take or most of the pharmacopoeias generally advise you to take about 1 gram of drug. Now this 1 gram of drug that you are going to take you are just going to take in atmosphere and it is bound to be contaminated with microorganisms. Now, this 1 gram of drug you are going to transfer to a flask.

containing sterile media. So, if you remember, maybe in your microbiology or biochemistry, you have this conical flask with non-absorbent cotton. Now, this should contain your sterile media, and in that, you are going to add your 1 gram of sample. Now, when you add your 1 gram of sample, you are going to shake it thoroughly. Why?

Because whatever microorganisms are there in your sample should get transferred to the media. Now, if the sample is very dense, you allow it to settle, or in some cases, even a

coarse filtration with sterile filter paper is recommended. Now, but that leads to what is called inaccuracy.

So, in some cases, it is filtered; in some cases, it is centrifuged. So, after centrifugation, you get a clear supernatant, and that supernatant is used. Now again depending upon the conditions in some cases where there are more lipophilic drugs, even surfactants to a little concentration of 0.1% may be added to facilitate the transfer of microorganisms to the media.

Now, once you have the sterile media, you kind of do what is called a serial dilution. Now, serial dilution, you already have 1 gram and, say, for example, 100 ml in media. So your concentration becomes 1%. Let's put it as 1%. Now, if you take 1 ml of it and

dilute it 10 times with just water or another sterile medium, the concentration will become 0.1%. Now, again dilute 1 ml of this. Make the volume 10 ml. You will get 0.01%. And then, finally, again take 1 ml of this.

Add around 9 ml of sterile medium. And you will get 0.001%. Now, why is this dilution important? You can see there are certain microorganisms, and from the samples, you cannot really make out visually by seeing it what the concentration is.

So, say, for example, if I have to plate it—that is, if I transfer this to a medium, say 1 ml here, 1 ml here of this, 1 ml here of this. So when I say my solution is concentrated, you see there is a film-like structure being formed. In that case, it becomes difficult for me to enumerate how many microorganisms are present. I further dilute it.
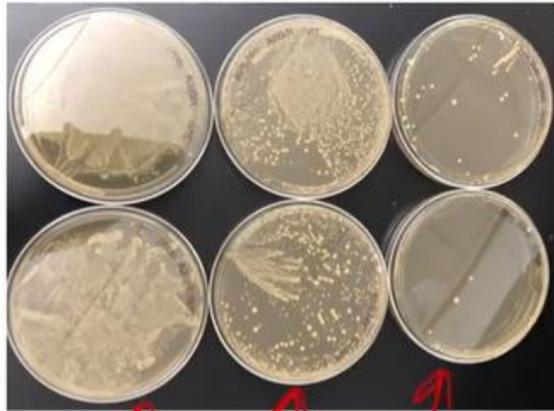
Plates showing microbial colonies
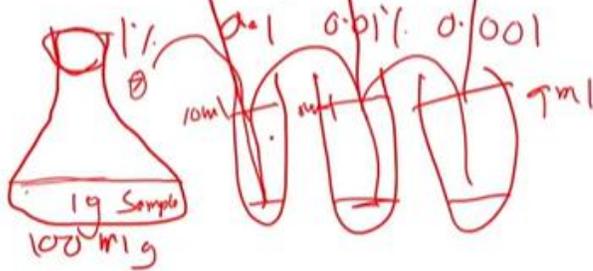
Now at least I am seeing some colonies. Now, what happens in your microbiology? Every microorganism which is suspended in your plate initially by a pour plate method or spread plate method will gradually give rise to one colony. So one colony initially was just one microorganism.

So these are called colony-forming units. So each CFU is considered to be one microorganism. Now, if you see the last dilution, this is the one I want because I can easily count how many are there. So if I say my 0.001 gram contains 4 colonies. Or 4 CFU to be specific, my 1 gram contains how much?

So I will just multiply it and I will get 400 CFU. 4000 CFU. So in this case, what I get is, if I say the limit is, for example, my drug should not contain more than 1000 CFU. So if 1 gram should not contain more than 1000 CFU and if my reading is higher than 1000 CFU. For example, 4000, that means my sample is failing the microbial contamination test.

But on the other hand, my limit says 1 gram should not contain more than 1000 CFU. If, due to some reason, my CFU is just 400 CFU. That means it is less than 1000 CFU per gram. So, what do I get in that case? What I get in that case is that it passes the test because 1 gram contains less than 1000 CFU.

So, I can do that. Now, in this case, I can also selectively bifurcate between the bacterial count and the fungal count. For my bacterial count, this is a little non-specific, which also allows certain fungi to grow. I can use my soybean casein digest media and incubate it at 37 degrees Celsius for a day, and I'll get my colonies. Whereas for fungus, I can use something more specific,

such as Sabouraud's dextrose agar, which is slightly acidic in pH and doesn't allow bacteria to grow. And this can be incubated. Fungi are preferentially grown at a slightly lower temperature. So mostly, you will see it incubated between 20 to 25 degrees Celsius. I can observe the same thing, but when it comes to fungal growth, it is more of a filamentous kind of growth that is seen.

Another method is to grow it in liquid media, referred to as broth. In that case, I will observe the turbidity. More the microorganisms, more will be the turbidity. So I measured light scattered by the organisms at about 600 nanometers and based on how much light is being transmitted, I can take a call whether the microorganism count is high or it is low

by comparing it with a standard optical density values. Now, coming on or moving on to the specific pathogenic microorganisms. Now, pharmacopoeias or WHO says, say, for example, acacia, one gram of acacia should not have more than 1000 CFUs. And we just saw we can do that. But when it comes to pathogenic organisms,

the pathogenic organisms are something which we do not even require one CFU to be there. One gram should not even have one CFU. In certain cases or in very rare cases, they do allow up to CFU, 10 CFU per gram, but that's not always the case. So pathogenic microorganisms, other microorganisms we want to get rid of. which we do not want to have in our herbal drugs.

And as a result, a good quality control method should selectively check if these microorganisms are present and to what extent they are present. So we can do that by harvesting or we can do that by allowing this dilutions to be poured on selective medias, which will allow us to check only for the presence of pathogenic microorganisms. So the selective medias are medias which will allow more of a pathogenic microorganisms to be alive, will kill all the normal or other non-pathogenic microorganisms or in certain cases it will still allow the other non-pathogenic microorganisms to grow but they will show a different color as compared to your pathogenic. So there might be a color difference or there might be a selectivity in growth. And such media are called as selective media.

So, for *E. coli*, more selective media are your sorbitol MacConkey media. Now, this is more for your... what you call it as lactic acid digesting bacteria so in this case whenever you have your sorbitol MacConkey media or eosin methylene blue agar plate they have a typical indicator say something like a phenol red so what is going to happen is first preferably your lactose will be digested to lactic acid

or fermentations will happen and more and more of this acids will be produced As more and more of this acid is produced, say, for example, by your E. coli, you will see that your phenol red will make it red. And as a result, your E. coli colonies will appear red, whereas other colonies might appear just white because they're not producing any acids.

Same applies with your eosin methylene blue, wherein your media causes acidity wherever your *E. coli* is growing. And that gives a little green metallic sheen. Now, coming to Salmonella, Salmonella specifically digest xylose. Then it kind of digest your lysine also. Now, after this, what happens is you will see this Salmonella kind of reacts with certain metals like your

Iron in this or in certain cases even lead and that gives rise to what are called as black centers or black colonies. So you will see a black centers or black colonies coming out of precipitation due to reaction with metal ions. When it comes to Shigella, you have Salmonella Shigella media, which is non-specific for both. That is Salmonella and both Shigella will grow. Again, that gives you a black color colonies or specifically Shigella broth,

which uses a triptych soy broth with yeast extract. Now coming to Suramunas, Eruginosa is more often an organism which is known to cause blindness. Now this can be detected selectively. The agar used for this is cetramide that is your cetyl trimethyl ammonium bromide agar. And what happens is very few organisms can stay or survive on this.

And as a result, not only will the pseudomonas stay on it, it starts producing different different pigments. These pigments include your piosinin and pioverdin. Now this piosinin and pioverdin gives you a little blue green and yellow colonies and as a result wherever the pseudomonas is growing the media or the plate is seen with greenish or greenish yellow pigments. So presence of this pigment itself in your cetramide agar is an indicative that your Pseudomonas aeruginosa is present.

The next one is the pneumonia causing *Klebsiella pneumoniae* which is selective media for this is your chrome agar. Now chromagar also contains your indicators or your pH change indicators and this gives you metallic blue color colonies on the reaction with certain indicators. So these are more selective medias. This will help us tell.

In certain cases, you will observe that when you see a total viable aerobic count, the incubation time is very short, maybe just 24 hours. But when you have to grow pathogenic microorganisms, pathogenic microorganisms are not commonly occurring organisms. So their concentration even in herbal samples is pretty low.

But still, you have to ensure that they are absent. In such cases, these herbal samples are taken and first grown in enrichment media. This enrichment media will allow selective growth of these pathogenic organisms. So all these organisms will be first grown, or the samples will be first treated with enrichment media for a day or two. So initially, for a day or two when the enrichment media is used,

you will see, preferably, your E. coli will stay, and the rest will die. Preferably, your Salmonella will stay, and the rest will die. So such selective enrichment media are used, and once the microorganisms are enriched, they are transferred to this plate. So comparatively even for the growth the incubation time lasts

between 24 to 48 hours, and in some cases, it goes up to 72 hours because their count is very low, and in order to make a good estimate of whether the microorganism is present or not, it is very vital to see the end results. So here are a few references which you can refer to in case you want to check your microbial growth, microbial media, your hemolysis, or different biological methods.

Like I said, efficacy testing is still there. But since efficacy testing is a bigger volume to handle, it alone is a big subject, considering or taking into account that different diseases require different animal models. To prove its efficacy, we will restrict ourselves to these biological methods. So, thank you everyone for your patient listening.

Thank you.