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Invited Review

Bioavailability Studies of Nutraceuticals

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ABSTRACT

Despite increasing interest in in nutraceuticals and their potential health benefits, not much is known about bioavailability of most of these nutraceutical compounds. Although they are considered dietary spplements and are subjected to a limited form of regulation, there is, however, a need to improve the efficacy and safety of these nutraceuticals. Additional research which defines the pharmacology, stability and bioavailability of these products is expected to gain strength and may offer a better understanding of their applicability in the prevention of disease conditions. This article reviews some aspects of nutraceutical bioavailability with examples from our work on the absorption studies of minerals from spirulina (*Arthrospira plantensis*) and gamma-oryzanol from rice bran (*Oryza sativa*) extract which emplyed human colon carcinoma (Caco-2) cell line and in vivo bioassays using animal models. Bioavailability of iron from spirulina was compared with its common source $FeSO_4$. Using the *in vitro* digestion protocol in combination with Caco-2 cell culture system, spirulina showed a high iron bioavailability compared to $FeSO_4$. The presence of other dietary factors (calcium, ascorbic acid, zinc, tannin and caffeine) was found to be not as significant as ferrous sulphate in affecting the iron uptake from spirulina. *In vivo* study showed that the efficacy of iron repletion in anaemic rats was enhanced in groups fed either commercial or cultured spirulina with improved haematological parameters of iron status. Further work on the behaviour and distribution of radiolabelled iron from spirulina has shown that iron-59 retained in the GIT of mice was lower in spirulina group compared to $FeSO_4$. Bioavailability study of gamme oryzanol was similarly conducted using Caco-2 cell as *in vitro* system and rabbit as *in vivo* model with the application of different formulations of gamma oryzanol in comparison with the natural form. Both systems showed that gamma oryzanol in its natural oil was poorly absorbed. However, when converted to other formulations, gamma oryzanol bioavailability was greatly increased by as much as 200 and 33 times more from the emulasion and microspheres respectively. These findings suggest that the efficacy of nutraceuticals in particular plant derived products which contain many ohytochemicals should be assessed in terms of not only their potential health benefits such as antioxidant action but also their bioavailability in order to provide a more wholesome picture of their potential.

INTRODUCTION

In the past decade, substantial progress has been made concerning our knowledge of bioactive components in plant foods and their links to health. Human diets of plant origin contain many hundreds of compounds which are not considered as nutrients at the present time, but appear to play a role in the maintenance of health or even in the alleviation of certain disease

conditions. These substances are referred to as nutraceuticals. The term 'nutraceutical', launched by the Foundation for Innovation in Medicine in 1989, defines a wide range of foods and food components with a claimed medical or health benefit, including the prevention and treatment of disease.[1] This terminology has brought out heated debate as it blurs the traditional dividing line between foods and medicine. Over the past five to ten years a large number of new food ingredients labelled as being nutraceuticals have been launched on the food and pharmaceutical markets. A market estimated at between US$6 billion and US$60 billion, growing at a pace of 5% per annum is a fast growing market worldwide.[2] The nutraceutical industry has made an impact not only in developed countries like the United States (US), Europe and Japan, but also in Malaysia. The Malaysian nutraceutical industry is growing equally fast at a steady pace of 20% per year . In 1996 the market was worth RM 60 million but recently it was reported that the market for herbal-based products alone has reached RM 2 billion. The beneficial action of nutraceutical ranges from the supply of essential minerals or vitamins to protection against several chronic diseases. While the health benefit of a nutraceutical may be a metabolic response that lowers risk of disease, the initial target for the food or food component may be on the functioning of the gastrointestinal tract (GIT).

IMPORTANCE OF BIOAVAILABILITY

Bioavailability is key to the effectiveness of nutraceuticals.[3] The total ingested amount of a nutraceutical normally does not reflect the amount that is available to the body through absorption. Only a certain amount is bioavailable. Bioavailability or biological availability is the term used to describe the proportion of a nutrient or active compounds in foods that can be utilised for various body functions. Traditionally, the most important consideration relating to nutrient bioavailability has been the attainment of an adequate nutritional status. However, lately nutraceutical containing minerals and vitamins as well as those containing other bioactive ingredients (eg. polyphenols, phytoestrogens, phytosterols) are being used by consumers for effects other than adequate nutrition.

Why is bioavailability thought to be so important? The Federal Food, Drug and Cosmetic Act (FFDCA; 1) does not provide a statutory definition of nutraceuticals; thus the Food and Drug Administration (FDA) has no authority to establish a formal regulatory category for such an item.[4] With the absence of specific regulatory categories for nutraceuticals, their regulation framework was not as rigorous and so bioavailability of nutraceuticals had often been overlooked. But with the increasing and relatively uncontrolled usage of nutraceuticals, issues of safety and efficacy were raising awareness of the need to look at bioavailability data. Thus, if 2 products claimed to have the same ingredients in the same quantities, but had different bioavailabilities (eg. different formulations) both safety and efficacy could be affected. On the one hand, high bioavailability was not always beneficial, simply because it could result in toxicity, but on the other, low bioavailability could result in a supplement that did not have the intended effect.

Bioavailability also has implications for the cost of an effective dose of a nutraceutical to the consumer. For example, the cost of a 30-day supply of iron supplement (providing 65 mg of iron a day) varied from 95 pence for ferrous sulphate to 146 pence for ferrous gluconate.[5] Study however had showed that the iron bioavailability was similar for both

the ferrous sulphate and ferrous gluconate.[6] In such a case other factors may be considered for consumer preference.

In addition, bioavailability also has implications for labeling of nutraceutical products. The concentration of a certain nutrient declared on the label, even if accurate, does not necessarily give an indication of its bioavailability. Variability in the content of the desired active ingredients in the harvested plant or food product will result in lack of predictability.[7] Here bioavailability could be one of the components that could be the key for standardisation of materials or other active compounds for nutraceutical development.

Methods of Assessing Bioavailability

There is limited information on bioavailability of nutraceuticals as the quantification of bioavailability is a difficult process. Generally the study of bioavailability can be subdivided into three constituent phases:[8]

1. availability in the intestinal lumen for absorption
2. absorption and/or retention in the body
3. utilisation by the body

First phase is dependent upon the processing and the form of the nutraceuticals for delivery. As for phases 2 and 3, homeostatic regulatory mechanisms and individual physiological needs of the body play the major role in determining nutraceutical bioavailability. Factors influencing bioavailability can be summarised in two main groups of diet-related and physiological factors (Table 1).

The various techniques that have been established to assess bioavailability can be broadly subdivided into *in vitro* and *in vivo* techniques.

Bioavailability Studies Using in vitro System

In vitro method provides a rapid and inexpensive way to measure bioavailability. From a logistical standpoint, it is not feasible to do all the needed testing on animal models and humans. It is neither likely nor anticipated that an *in vitro* method will be able to predict precisely how much of a nutraceutical will be absorbed and utilised by a human subject. However, this method serves as a preliminary screen to identify the most promising concentration and component to be further tested on animal or human models.

In vitro studies range from measurements of solubility,[9] dispersibility[10] and dialysability[11] to the studies of molecular uptake in cell culture model.[12,13] However, it has been found that each method used in these measurements lacked some of the versatility, visibility or both, needed to present the intact viable intestinal mucosa.[14] For example, in transport studies using everted sacs and intestinal loop, the rate of disappearance from luminal side is assumed to be equal to the uptake rate.[14] In everted sacs and intestinal loop, the compounds that bind to the cell surface or the intracellular sites after uptake, might undergo metabolic biotransformation in the lumen or during transepithelial transport. For these nutraceutical compounds the disappearance from the lumen does not equal the amount absorbed, which results in an overestimation of the true absorption rate. A better estimation of absorption rate can be done using vascularly perfused intestine since it enables the sampling

of blood or lymph from mesenteric veins and lymph ducts. However, this system does not provide information on the processes involved in the transmucosal transport of nutraceutical molecules.[14]

The development of human cell culture systems has been limited by the lack of retention of anatomical and biochemical features of differentiated cells *in vivo*.[15] On the other hand, several human colon carcinoma cell lines namely, Caco-2, HT-29, SW1116 and LS174T have been reported to undergo enterocytic differentiation in culture.[16] Recently attention has turned to Caco-2 cell line, which appears to have the most highly differentiated properties under standard culture conditions.[17,18] These cells, under appropriate conditions, differentiate into polarized enterocyte-like monolayers and in many ways, act similarly to small intestinal epithelial cells.[19] Nevertheless, the main disadvantages of *in vitro* models include the lack of complex mucosal barrier with all of its regulatory processes and also the static transport conditions not allowing an accurate calculation of whole fractional transport and flux rates.[20]

In our laboratory, human colon carcinoma (Caco-2) cell line has been used for the estimation of nutraceutical bioavailability. The cell line is grown as a monolayer on polycarbonate membrane in transwell bicameral chambers. In this system, the apical and basolateral culture fluids are separated allowing the transport of molecules from one culture fluid to the other to be determined (*Fig. 1*).

Bioavailability Studies Using in vivo System

In vivo bioassays not only complement *in vitro* studies but also provide additional information on bioavailability of nutraceuticals by using the response of the experimental animal or man to these compounds. Direct determination of the active compounds and their metabolites in the tissues, blood, urine or of the enzyme levels may provide data of its utilisation. The use of radio and stable isotopic techniques for some nutraceuticals further allows the investigation of its route and tissue distribution. Animal model is normally used to study the bioavailabiltiy of active components that are completely metabolised in the body. This is because blood sample is needed since the compounds cannot be detected in the urine. Although human trial is preferable, this test is not convenient for human subjects since blood samples should be taken around 10 times a day and is therefore used only for confirmatory test at the end of the investigation. In this study set up, a single dose is administered orally, after which the total area under the curve in plasma versus time curve (AUC) reflects the amount of the compound reaching the bloodstream. The AUC after oral administeration compared to that obtained after intravenous administration, the fraction (F) of the oral dose available to the systemic circulation can be determined using the formula $F = (AUC)_{oral}/(AUC)_{iv}$.[21] Nevertheless the intravenous dose must be smaller than the oral dose to achieve comparable blood levels. In this case, different oral and intravenous doses are used for estimating systemic bioavailability, using $F = (AUC)_{oral} D_{iv}/(AUC)_{iv} D_{oral}$, where D refers to the dose.[21] Since most bioactive compounds are not approved for intravenous use, the absolute bioavailability has not been determined for many of these compounds. Hence, the bioavailability is usually determined against a relative standard which is normally an aqueous oral solution of the compound, and in this case, relative availability (F_{rel}) =

$(AUC)_{test}$ /$(AUC)_{standard.}$ Some compounds cannot be formulated in aqueous dosage forms, and therefore other forms like non aqueous oral solutions, oral suspensions, or other solid oral dosage forms can be used as standards. In this case it is not uncommon to find that $F_{rel} > 1.0$.[21]

Other *in vivo* techniques using radioisotope and stable isotope balance are also used in assessing nutraceutical bioavailability.[19] These techniques estimate the bioavailability in a mass balance-like fashion from the apparent absorption by determining the differences between the fed and the excreted amount of the active compound or nutrient. Since the introduction of stable isotopes, stable isotope balance technique has been used extensively in bioavailability studies.[22,23,24]

We have carried out some work on the bioavailability of nutraceuticals in particular iron supplements from spirulina (*Arthospira platensis*) and gamma oryzanol from rice bran extracts using both *in vitro* and *in vivo* experimental models. The effect of dietary factors, different nutraceutical formulations and molecular interactions are the focus of the studies.

Bioavailability of Iron from Spirulina

In the natural product industry plants are being cultivated for their use as nutraceutical resources targeted for improved health and pharmaceutical products.[25] One such example is the use of spirulina as a source of minerals like iron. Iron malnutrition has been known to be a worldwide public health problem with disastrous functional consequences affecting women and children. The rapid growth of mineral supplement industry is in part due to the need for Fe supplements in alleviating these health problems especially in diets lacking adequate mineral content. Nonetheless, depending on the nature of the supplements, they may not provide minerals in a soluble and metabolically available form for optimal utilisation.[26] The poor performance of the mineral-based supplements, particularly the multivitamins in providing the minerals in soluble form indicates that these supplements do not provide their minerals in the desired bioavailable form.[7]

Iron bioavilability from spirulina using three different methods namely *in vitro* digestion/ Caco-2 cell culture method, haemoglobin repletion assay and radioiron balance study demonstrated the high availability of iron from spirulina.[27] Using the *in vitro* digestion protocol in combination with Caco-2 cell culture system, spirulina showed a high iron bioavailability compared to $FeSO_4$ and this result was in agreement with the study done on iron-fortified spirulina by Puyfoulhoux and co-workers utilising similar method.[28] Two different *in vitro* digestion methods were applied for this study, namely dialysis and centrifugation procedures. Centrifugation method yielded significantly higher iron uptake for spirulina compared to the dialysis method. On the contrary, $FeSO_4$ was not affected by either the dialysis or centrifugation methods (*Fig. 2*). The presence of other dietary factors (calcium, ascorbic acid, zinc, tannin and caffeine) was found to be not as significant as ferrous sulphate in affecting the iron uptake from spirulina, suggestive of competitive behaviour of the non-haem iron component of spirulina.

In vivo study on rats proceeds the Caco-2 cell culture system to further investigate the functional consequence of Fe bioavailability from spirulina and $FeSO_4$. Haemoglobin, and haematocrit levels of rats fed both spirulina and $FeSO_4$ was found to be comparable. The calculated percentage of haemoglobin regeneration efficiency (HRE) however showed that

efficiency of iron absorption from spirulina was better than $FeSO_4$ (Table 2). The presence of calcium did not significantly affect iron availability in both spirulina and $FeSO_4$.

In another study conducted using iron deficient mice fed either spirulina or $FeSO_4$ it was found that the percentage of iron-59 retained in the GIT of mice was lower in spirulina group compared to $FeSO_4$. This might be due to the amount of fibre present in spirulina which could fasten the transit time of the food that passes through the GIT. Even though the amount of iron-59 retained in mice-fed spirulina was lower than those fed $FeSO_4$, the amount of iron-59 being absorbed by the iron deficient mice was comparable both in the spirulina and $FeSO_4$ groups at 6 h and 24 h intervals. At 7 d, mice fed $FeSO_4$ showed a significantly higher amount of ^{59}Fe absorbed compared to mice fed spirulina. The results indicated that spirulina might be more efficient in repleting iron deficient conditions in a short period of time (6 h and 24 h). In contrast, for the iron normal group, mice fed $FeSO_4$ showed a better absorption of iron-59 at 6 h and 24 h compared to mice fed spirulina which postulates the protective role of spirulina in excess iron intake. In the presence of calcium, iron absorption in iron deficient as well as iron normal mice fed both $FeSO_4$ and spirulina was not significantly inhibited.

In conclusion, this study indicated that spirulina is a promising source of iron supplement and also iron fortificant. It is a concentrated source of iron suitable for both the iron normal and iron deficient states. In iron normal state, its selective release mechanism could prevent iron overload and toxicity. This is especially important in people with HFE mutation and elderly persons who might be at risk of iron overload. In an iron deficient state, it is highly bioavailable and is thus easily absorbed by the body. Besides that, evidence for its potential therapeutic application is overwhelming in the areas of immunomodulation, anti-cancer, anti-vital and cholesterol reduction effects.[29,30,31,32,33]

Bioavailability Studies of Gamma-oryzanol

Cardiovascular disease is now the major cause of mortality in many countries. Observation studies proved that there is a proportional relationship between plasma lipid levels and cardiovascular disease-induced death rate [34]. Current research has therefore focused on the search to reduce plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL) and triglyceride (TG) both by pharmacological and dietary means. It has been emphasized that the dietary intake of low cholesterol food, mono and polyunsaturated fats, dietary fibers, fruit, and vegetables helps to minimise blood cholesterol level.[35] Among other dietary ingredients, which are helpful for the serum cholesterol reduction, plant sterols were found to be an effective dietary additive.[36] In addition to tocopherols and tocotrienols, rice bran oil is among grains that contain plant sterols. Rice bran oil and its components have demonstrated a healthy effect for many human diseases, especially in their hypocholesterolemic effect. Other potential properties of rice bran oil and its components include anticancer effect, modulation of pituitary gland secretion, and inhibition of platelet aggregation.[37]

Rice bran is made from the pericarp and the germ of rice grain. It constitutes about 10% of rough rice grain and contains around 20% oil.[38] In contrast to most common refined vegetable oils, crude rice bran oil contains a rich unsaponifiable fraction (up to 5%), which consists of sterols (43%), triterpine alcohols (28%), 4-methylsterols (10%) and 19% less

polar components.[39] Phytosterols include ß-sitosterol (900 mg%), campesterol (500 mg%), stigmasterol (250 mg%), squalene (320 mg%) and g-oryzanol (1.6%) which is a mixture of ferulic acid esters of triterpine alcohols such as cycloartenol (106 mg%) and 24-methylene cycloartanyl (494 mg%)[40,41] (Metwally *et al.* 1974; Norton 1995) first isolated by Kaneko and Tsuchiya in the early 1950s.[42] Its fundamental molecular structure is the ferulic acid aromatic phenolic nucleus esterified to cyclopentanperihydrophenanthrene.[43] Rice bran oil contains 72-612 ppm tocotrienols[44] and 100 mg% tocopherols.[45]

Health Benefits of Rice Bran Oil and Its Components

Rice bran oil is rich in unsaturated fatty acids. Studies have shown that a diet containing a high percentage of saturated fatty acids increases serum total cholesterol while those enriched with unsaturated fatty acids can decrease serum total cholesterol, LDL-cholesterol and increase HDL- choleterol.[37] Many studies have been carried out to study the effect of rice bran oil on lipid metabolism. It has been found that rice bran oil lowers total cholesterol, VLDL-cholesterol, LDL-cholesterol and HDL-cholesterol.[46] In addition to the effect of unsaturated fatty acid in reducing plasma cholesterol, unsaponifiable sterols including oryzanol (*Figure 3*) have significant effects on LDL cholesterol level.[47] In addition to its hypercholesterolemic effect, oryzanol has been found to have curative effect for many diseases including inhibition of platelet aggregation, antistress action, and has been used for the treatment of psychosis and various disorders of the autonomic nervous system.[37,48]

Studies have shown that phytosterols have limited bioavailability due to poor absorption from the GIT and fast excretion from the liver.[49] Presently, there is little information available regarding the absorption and distribution of phytosterols. We have therefore through our rice bran research programme conducted bioavailability studies of gamma oryzanol. In the *in vivo* study, rabbits were used as the animal model in which different oryzanol formulations were administered orally and compared with the intravenous dose, which was administered in emulsion form. *Figures 4* and *5* represent the results of using both *in vitro* and *in vivo* systems. Results showed that gamma oryzanol in its natural form were poorly absorbed. However, the absorption was significantly improved from other formulations. Our findings support Heinemann and co-workers[50] who found that absorption rate of phytosterol was less than 5% of dietary levels, which is lower than cholesterol that can exceed 40% despite the similarity in their structures. Gamma oryzanol structure is different from cholesterol by esterification to ferulic acid. Inadequate esterification of phytosterol has been suggested to be a reason for its poor absorption.

Insufficient water solubility of gamma oryzanol could further add to its poor absorption.[51] It is necessary for a compound to dissolve in the aqueous fluid of the GIT before being absorbed while being sufficiently lipophilic to penetrate the lipid membrane of the gut.[52] It was found that micellarisation is a very important step in the absorption of lipophilic compounds since it increases the amount available to be absorbed.[53] Since dietary mixed micells has limited capacity to solubilise lipophilic molecules,[54] thus preparation of oryzanol emulsion helps to improve its absorption through cellular barrier. Microparticulate drug delivery systems are now used as carriers for lipophilic compounds to improve their therapeutic efficacy.[55] In our study oryzanol loaded microspheres showed enhanced bioavailability in comparison with the natural form. Polymeric materials have the capability

to improve the therapeutic efficacy of many compounds due to their large size molecules which enable them to stay at the delivered site for a long time.[56]

In conclusion, emulsion could be considered a good alternative dosage form for oryzanol since it enhances its bioavailability around 200 times. The addition of vitamin E to the emulsion will improve its physiological effect, because vitamin E, especially tocotrienol has positive effect on plasma HDL-cholesterol while oryzanol has no significant effect.[57] On the other hand, the addition of vitamin E to the oryzanol emulsion can be considered a natural preservative that improves its stability and shelf life. Oryzanol loaded microspheres is an alternative formulation to oryzanol emulsion for those who are not agreeable with liquid formulations. In addition, oryzanol emulsion and microsphere formulations provide adequate and sustained oryzanol concentration in plasma for effective physiological function.

CONCLUSION

The evolving concept of nutraceuticals raises exciting prospects for biotechnology and in particular nutrition research in line with the country's initiatives in exploiting local resources for health produts development. Despite the vast interest in nutraceuticals and their diverse roles in human health, little is known about the transfer of many of these active compounds from foods to micelles or aggregates during digestion and subsequent transfer across the intestinal mucosa for absorption. The permeability of these active compounds as well as their bioavailability after ingestion could be the limiting property for some of them. As a consequence, their potential health promoting properties may be jeorpadised. Improved knowledge of the metabolic fate of the active compounds in terms of tissue uptake and retention will assist in the development and testing of hypotheses regarding their potential to influence biological processes in the human. Both *in vitro* and *in vivo* methods may be employed to assess the bioavailability of these nutraceuticals. However, *in vitro* cell culture model in particular Caco-2 cell culture system is useful for a better understanding of the mechanisms involved in the absorption and fate of the nutraceuticals at cellular level. Hence, detailed knowledge of nutraceuticals bioavailability will be an important pursuit of researchers for many years to come. For consumers to agree to pay the premium associated with nutraceuticals, they must be convinced that their health claim messages are clear, truthful and unambiguous. Safety, quality and cost-effectiveness must remain paramount. A well informed health-conscious public will ensure that nutraceuticals follow vitamin supplements into long mature life-cylcles for both the product manufacturer and the consumer.

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