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Mini Review

Medicinal plants and antioxidants: What do we learn from cell culture and *Caenorhabditis elegans* studies?

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ABSTRACT

Traditional medicinal plants have a long history of therapeutic use. The beneficial health effects of medicinal plants rich in polyphenols are often attributed to their potent antioxidant activities, as established *in vitro*, since diets rich in polyphenols are epidemiologically associated with a decreased incidence of age-related diseases in humans. However, medicinal plants may also exert pro-oxidant effects that upregulate endogenous protective enzymes. Care is needed when studying the biological effects of medicinal plants in cell culture because some polyphenols oxidize readily in culture media. This review summarizes the data we have obtained from *in vitro* and *in vivo* (*Caenorhabditis elegans*) studies examining the diverse effects of traditional medicinal plants and their modes of action.

1. Introduction

Traditional medicine is increasingly popular as a cost effective alternative to, or complementary to, orthodox medicine. According to the World Health Organization (WHO), 80% of the population in some Asian and African countries depend on traditional medicine for primary health care needs. Traditional medicinal products constitute multi-billion-dollars industries worldwide.

Traditional medicinal plants, also known as herbal medicines, botanical medicines or phytomedicines, refer to the medicinal products of plant roots, stems, leaves, bark, seeds, fruits and flowers that can be used to promote general health and treat diseases. These different products of the plants may be used directly in a prescription formula or processed into different ready-to-use products. Traditional Chinese medicine (TCM) has long been used to maintain well-being and treat or prevent diseases [1–3]. TCM does not cure chronic diseases directly but it tries to restore the body to a normal state by balancing the five elements in our body and to grant vital energy, or "qi", which has both Yin and Yang aspects [3,4]. An imbalance between stress and protective elements *in vivo* is suggested to play a role in disease development [5]. Therefore, TCM might play a role in disease prevention by promoting the body's resistance to disease, and its "self-recovery".

2. Medicinal plants as antioxidants

Several epidemiological studies have found an inverse association between the intake of diets rich in polyphenols (such as fruits, vegetables, and grains) and the risk of age-related diseases in humans [6–9]. This association is often attributed to the powerful antioxidant activities of flavonoids and other polyphenols, as established *in vitro*, to scavenge a wide range of reactive oxygen, nitrogen, and chlorine species [10–12]. An antioxidant is defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" [5]. Therefore, antioxidants may serve to control the levels of free radicals and other "reactive species" (RS) to minimize oxidative damage.

There is an abundance of anecdotal evidence on protective effects of medicinal plants (MPs). Some of these effects include antidiabetic, antimicrobial, antiviral, anti-inflammatory, antiallergic, immunosuppressive, immunostimulatory and cancer chemoprevention effects [13–16]. These beneficial effects are often assumed to be acting by antioxidant mechanisms because MPs are rich sources of polyphenols. As examples, the Trolox equivalent antioxidant capacity (TEAC) values of 112 MP extracts correlated well with total phenolic content and a linear relationship between oxygen radical absorbance capacity (ORAC) values and total phenolic contents has been reported in medicinal and culinary herbs [17,18]. In our own work, we showed that the TEAC values of selected MP extracts correlated well with total phenolic contents and 2,2-diphenyl-1-picrylhydrazyl (DPPH) reducing abilities [19]. Moreover, we have given special attention to a semi-purified extract (YCT) with powerful antioxidant properties from *Cratoxylum*

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cochinchinense (WN, highest TEAC value among our MPs), because it effectively scavenged RS such as hypochlorous acid (HOCl), superoxide radicals (O_2^-), peroxynitrite ($ONOO^-$), and inhibited lipid peroxidation and formation of advanced glycation end products [19].

3. Medicinal plants as pro-oxidants

Although MPs have been widely acclaimed for their antioxidant effects, there is increasing evidence pointing to pro-oxidant effects [19–22]. MPs are rich sources of polyphenols and some of these polyphenols have been shown to oxidize readily in beverages such as green tea [23,24]. TCM prescription formulae often involve a decoction process with water of a mixture of MP products for several hours. Polyphenols in the MPs may oxidize during the preparation and storage of these TCM decoctions and produces RS such as O_2^-, hydrogen peroxide (H_2O_2) and a mixture of semiquinones and quinones. Contamination or adulteration of MPs with heavy metals including transition metal ions such as iron and copper can aggravate the pro-oxidant effects of MPs by catalysing oxidation reactions as well as by Fenton chemistry [5]. Besides, polyphenols are typical xenobiotics that are metabolized by phase I (cytochromes P450) and phase II (glutathione transferases, glucuronyl transferases and sulfotransferases) enzymes [5,25]. The metabolism of xenobiotics/polyphenols can also produce RS, reactive intermediates and metabolites that can act as pro-oxidants. Indeed, we have shown that MP extracts induced oxidative stress in various cell types as determined with fluorescent probes for measuring different RS [26]. For example, several RS can be detected with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), O_2^- with dihydroethidium (DHE), reactive nitrogen species (RNS) with 4-amino-5-methyamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA), lipid peroxides with *cis*-parinaric acid and C-11-BODIPY$^{581/591}$.

However, one must be cautious on the interpretations of what these fluorescent probes are measuring and the possible artifacts that may occur [26,27]. Depending on the degree of oxidative stress and cell types, multiple biological responses are possible. For instance, the most powerful "antioxidant" extract, YCT, was found to be cytotoxic to several cell types, in part by inducing severe oxidative stress [28]. However, cells exposed to MP extracts at low concentrations and short exposure times (6 h or less) often showed increased cell viability as determined by the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [28–30]. In Jurkat T cells, YCT-induced cell death with features of both apoptosis and necrosis involved rapid rises in oxidative stress that affected calcium metabolism and mitochondrial function [28]. *Notopterygium forbesii* Boiss (NF) extract also induced rapid increases in RS in human fetal hepatocytes (HFHs) and RAW 264.7 macrophages [29,31], while *Psoralea corylifolia* L. (PC) extract-induced oxidative stress in SHSY-5Y cells was associated with inhibition of the mitochondrial complex I and proteasome activities [30]. Other powerful natural "antioxidant" extracts that induced oxidative stress in various cell types include *Cosmo caudatus* (UR), star fruits, pine bark and cocoa (unpublished data).

However, pro-oxidant effects are not necessarily bad, in fact, mild pro-oxidant effects may be beneficial [5]. The "right" levels of RS generated by oxidation and metabolism of polyphenols can up-regulate the levels of endogenous cytoprotective enzymes such as antioxidant defenses and xenobiotic-metabolizing enzymes [5,32]. For example, many of our MP extracts caused heme oxygenase-1 (HO-1) up-regulation in HFHs (unpublished data); in particular, the NF extract increased HO-1 protein levels in HepG2 cells, vascular smooth muscle cells, colon cancer cells (HT-29), human acute monocytic leukemia cells (THP-1) and RAW 264.7 macrophages. Up-regulation of HO-1 has been demonstrated to confer protective effects against oxidative stress-induced damage both *in vitro* and *in vivo* [33–35]. Indeed, silencing of HO-1 with siRNA in RAW264.7 macrophages decreased the inhibitory effects of NF and PF (phenethyl ferulate, an active compound isolated from NF) on lipopolysaccharide-induced up-regulation of cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) (unpublished data). The inhibitory effects of NF and PF were also decreased in the presence of *N*-acetyl-L-cysteine (NAC) and at the same time, NAC also reduced NF- or PF-induced increases in ROS and HO-1 protein levels [31]. Since NAC is a thiol antioxidant and redox modulator that removes ROS, its effect suggested that ROS can sometimes be anti-inflammatory at sites of chronic inflammation [31,36–38]. Other extracts (e.g. *Cortex magnoliae officinalis* (D), *Rhizoma chuanxiong* (G), NF and PC) also increased NADP(H)-quinone oxidoreductase 1 (NQO1) activity in Hepa1c1c7 mouse hepatoma cells (unpublished results). NQO1 is a measure of the induction of quinone reductase, which is one of the essential phase II detoxifying enzymes in the adaptative stress response [32]. In addition, many of our MP extracts increased the accumulation of polyubiquitinated proteins in HFHs and HepG2 cells (unpublished results), suggesting that they inhibited the proteasome system. Ubiquitin is a heat shock protein with molecular mass of 8500, whose levels rise 5–7-fold after stress [39]. The induction of catalase and superoxide dismutase (SOD) upon exposure to H_2O_2 in microorganisms, such as *Escherichia coli* or *Salmonella typhimurium* is another example of a beneficial protective effect due to mild stress [40].

4. Medicinal plant studies in *Caenorhabditis elegans*, an *in vivo* system

C. elegans is a small nematode (adults reach ~1.2 mm in length) that offers several advantages over other model systems such as *Drosophila* and rodents in studying effects on lifespan [41,42]. We examined the relation of the antioxidant activity of MP extracts, as established *in vitro*, with lifespan extension in *C. elegans*. Our results show no clear and direct correlation between the two, as well as complex actions. For example, PC extract at lower concentrations significantly extended lifespan but was toxic at higher concentrations [43].

5. Cautions on using cells in culture for oxidative stress studies

Cell culture is one of the most popular and commonly used model systems to study the cellular effects of MPs and chemical constituents isolated from them. However, most cells in the human body (except corneal, skin and respiratory tract lining cells) are exposed to O_2 concentration in the range of 1–10 mm Hg. Cells cultured under laboratory conditions of 95% air/5% CO_2 experience about 150 mm Hg of O_2. Therefore, these cells are constantly under oxidative stress because the rate of ROS production from cellular enzymes (such as xanthine oxidase and mitochondria) will increase if O_2 levels are raised [27,44–47].

MPs are rich sources of polyphenols; polyphenols are unstable and can act as pro-oxidants in cell culture media. Several artifacts that can occur in cell culture studies have been reviewed [5,20,27,48–51]. Therefore, it is important to check that the observed biological effects on cells in culture treated with various MP extracts are not due to oxidation of phenolic compounds in cell culture media. This should be verified by measuring stability of the polyphenols and by H_2O_2 production in the cell culture media (without cells) using either the O_2-electrode method or by FOX 2 assay [52,53]. Indeed, many of our MP extracts (50 μg/mL) were found to generate about 20 μM of H_2O_2 as determined using the FOX 2 assay under normal cell culture conditions [Dulbecco's

Modified Eagle's Medium (DMEM) + 10% fetal bovine serum + 1% penicillin and streptomycin, 37 °C, 24 h] (unpublished results). H_2O_2 generation rates can be affected in complex ways when two or more antioxidants are present [54]. Catalase (~500–1000 U/ml) can be added to the media to scavenge H_2O_2 generated by oxidation of test compounds. Our group has shown that some phenolic compounds are more stable in other culture media such as F-10 and F-12 as compared to DMEM [55]. Therefore, these media can sometimes be used to replace DMEM to minimize artifacts in cell culture experiments. Pyruvate in some cell culture media can scavenge H_2O_2; thus the absence of H_2O_2 generation in such media does not mean that an MP is not oxidizing [56].

6. Toxicity of medicinal plants in cell culture and *C. elegans*

Toxicity related to the use of crude MPs or products from MPs has been reported [57,58]. This can be due to contamination with toxic chemicals from cultivation, or during post-harvest processing and storage, misuse and confusion of medicinal plants, or adulteration [57,59]. Indeed, TCM has a comprehensive documentation (mostly in Chinese) of medicinal plants with unfavorable side effects [57,60]. To study the effect of MP extracts in cell culture, we suggest testing the extracts with a wide range of cell types because cytotoxicity is, to a certain degree, cell-type-specific [28]. However, PC extract was non-selectively toxic to both normal and keloid fibroblasts and keratinocytes (unpublished results). Also, extracts often inhibit mitochondrial complex I activity in mitochondria and ATP synthesis driven by this complex. Whereas several MP extracts inhibited at least one of the three proteasomal hydrolytic activities (peptidyl-glutamyl-peptide hydrolyzing, chymotrypsin-like and trypsin-like) in various cell types by 20–50%, others (such as WN, UR and pine bark) dose-dependently increased cellular proteasome activities (unpublished results). Whether the latter effect is due to mild oxidative stress is unknown [61].

In TCM, it is claimed that a composite prescription formula with a mixture of toxic (different levels of toxicity) and non-toxic MPs can improve the protective effects of the treatment and suppress the adverse effects from the toxic MPs [3,57]. However, a decoction derived from a mixture of MPs is chemically complex, and effects (beneficial or otherwise) could arise from many constituents or mixtures of constituents. Such complexity can hinder the progress in dissecting the mechanisms of action of MPs, both chemically and biologically. *In vivo* toxicity of PC in humans needs to be investigated by measuring well established biomarkers and with suitable controls [20,62].

7. Pitfalls in medicinal plant studies

MPs usually contain hundreds if not thousands of chemical ingredients that interact to give complex effects. Several of these compounds may interfere with the assays used for screening "bioactive" chemical entities or elucidating signaling pathways. For examples, extract from the seeds of PC interfered with the fluorescent measurement at excitation and emission wavelength of 350 and 420 nm, respectively, of advanced glycated end products [19]. This is probably due to the presence of furanocoumarins, such as psoralen and angelicin in the seeds of PC, which are active photosensitizing compounds widely used in photochemotherapy [63]. Other than interfering with fluorescence measurement due to the presence of strongly-colored compounds, precipitation of some compounds may occur at high concentrations, which can affect absorbance measurement.

When using fluorescent probes for measuring oxidative stress, PC extract was found to interfere with real time *in situ* RS measurement in SH-SY5Y cells loaded with H_2DCFDA in a fluorescence microplate reader [30]. Another extract, NF, at concentrations of 40 μg/mL and higher also interfered with DCF fluorescence measurement using a microplate reader [29].

Other compounds may have quenching effects on the fluorescent probes or the generated fluorescent intermediates. We have experienced this effect with 7-oxo-benz[*d*]anthracene-3,4-dicarboxylic acid, a metabolite of benzo[*a*]pyrene. This compound quenched both the green monomers and the red aggregates of JC-1 dye in Jurkat T cells and HCT116 (unpublished observation). JC-1 is a cationic dye that is concentrated in respiring mitochondria and it is widely used to study mitochondrial membrane potential in cells and isolated mitochondria.

In *C. elegans* lifespan or toxicity studies, results may be confounded by the "unpleasant" scent or taste of the MPs as the worms were sometimes observed to "run-off" to the side of the agar dish and die from starvation or dehydration. Pharyngeal pumping rate should be determined to ensure that the worms are not consuming less, and thus being subjected to caloric restriction (which can itself affect lifespan), to avoid the "unpleasant" test compounds [64,65].

The reductionist approach of isolation and elucidation of bioactive chemical constituents for pharmaceutical interest is not always appropriate for MPs [3,66]. This is especially true when the "bioactive" constituents in the plants are proanthocyanidins. Proanthocyanidins are essentially polymer chains of flavonoids such as catechins. The degree of polymerization depends on plant species and methods of extraction [67]. These compounds are challenging to purify (personal experience) and interesting to study. We found them in our plant extracts such as YCT, pine bark and extract UR (unpublished data and [68,69]), and others have reported them in, for example star fruits [70], berries [71], grape seeds [72] and cocoa [73]. Many beneficial effects of proanthocyanidins have been claimed [67,74] but not yet rigorously proven to occur.

8. Conclusion

Although little evidence supports the view that polyphenols are antioxidant or pro-oxidant *in vivo* in the human [49,75], our foray into this field revealed to us that some of the beneficial effects of polyphenol-rich MPs might be explained by their pro-oxidant effects rather than their "powerful" antioxidant activities. The former in turn increase the endogenous protective enzymes by exerting mild oxidative stress. Nevertheless, other potential biological effects of MPs are possible. MPs are chemically complex and the mechanisms responsible for their protective effects probably involve complex synergistic interactions and are challenging to elucidate. Care is needed to avoid artifacts when studying effects of MPs in cell culture and other model systems, and when fluorescent probes are used. It is important to understand that *natural* does not equate to *safe*. Proper use of MP products may provide therapeutic benefits but indiscriminate or excessive use can be unsafe and even dangerous. Due to a tremendous interest in MPs in both scientific and consumer circles, well-designed studies to investigate their biological effects *in vivo* in humans are urgently needed.

Conflicts of interest

The authors declare no conflicts of interest.

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