Review

Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. (Araliaceae) as an adaptogen: a closer look

Marina Davydov ¹, A.D. Krikorian *

Department of Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY 11794-5215, USA

Received 20 May 1999; accepted 17 December 1999

Abstract

The adaptogen concept is examined from an historical, biological, chemical, pharmacological and medical perspective using a wide variety of primary and secondary literature. The definition of an adaptogen first proposed by Soviet scientists in the late 1950s, namely that an adaptogen is any substance that exerts effects on both sick and healthy individuals by ‘correcting’ any dysfunction(s) without producing unwanted side effects, was used as a point of departure. We attempted to identify critically what an adaptogen supposedly does and to determine whether the word embodies in and of itself any concept(s) acceptable to western conventional (allopathic) medicine. Special attention was paid to the reported pharmacological effects of the ‘adaptogen-containing plant’ Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. (Araliaceae), referred to by some as ‘Siberian ginseng’, and to its secondary chemical composition. We conclude that so far as specific pharmacological activities are concerned there are a number of valid arguments for equating the action of so-called adaptogens with those of medicinal agents that have activities as anti-oxidants, and/or anti-cancerogenic, immunomodulatory and hypocholesterolemic as well as hypoglycemic and choleretic action. However, ‘adaptogens’ and ‘anti-oxidants’ etc. also show significant dissimilarities and these are discussed. Significantly, the classical definition of an adaptogen has much in common with views currently being invoked to describe and explain the ‘placebo effect’. Nevertheless, the chemistry of the secondary compounds of Eleutherococcus isolated thus far and their pharmacological effects support our hypothesis that the reported beneficial effects of adaptogens derive from their capacity to exert protective and/or inhibitory action against free radicals. An inventory of the secondary substances contained in Eleutherococcus discloses a potential for a wide range of activities reported from work on cultured cell lines, small laboratory animals and human subjects. Much of the cited work (although not all) has been published in peer-reviewed journals. Six compounds show various levels of activity as anti-oxidants, four show anti-cancer action, three show hypocholesterolemic activity, two show immunostimulatory effects, one has choleretic activity and one has the ability to decrease/moderate insulin levels, one has activity as a
radioprotectant, one shows anti-inflammatory and anti-pyretic activities and yet another has shown activity as an antibacterial agent. Some of the compounds show more than one pharmacological effect and some show similar effects although they belong to different chemical classes. Clearly, *Eleutherococcus* contains pharmacologically active compounds but one wishes that the term adaptogen could be dropped from the literature because it is vague and conveys no insights into the mechanism(s) of action. If a precise action can be attributed to it, then the exact term for said action should obviously be used; if not, we strongly urge that generalities be avoided. Also, comparison of *Eleutherococcus* with the more familiar *Panax ginseng* C.A. Meyer (Araliaceae), ‘true ginseng’ has underscored that they differ considerably chemically and pharmacologically and cannot be justifiably considered as mutually inter-changeable. Accordingly, we recommend that the designation ‘Siberian ginseng’ be dropped and be replaced with ‘*Eleutherococcus*’. In the case of both *Eleutherococcus* and true ginseng, problems inherent in herbal preparation use include inconsistencies not only in terms of indications for use, but in the nomenclature of constituent chemical compounds, standardization, dosage and product labeling. Finally, our re-examination and fresh interpretation of the literature on *Eleutherococcus* and comparison with true ginseng shows that the potential for a scientifically more complete and defensible exploitation of these plants will be better served by investigating and considering them in a context that consciously ignores the fact that the word ‘adaptogen’ was ever invented. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords:** Adaptogen; Adaptogenic activity; *Eleutherococcus senticosus*; *Panax ginseng*; Lignans; Anti-cancer agents; Anti-oxidants; Placebo effect; Saponins; True ginseng; ‘Siberian ginseng’

### 1. Introduction

There has been a dramatic revival in recent years in the use of herbal preparations for the treatment of a wide range of ailments (Eisenberg et al., 1998). Some products are being recommended for use as ‘specifics’ to treat particular illnesses or conditions much like conventional synthetic medications are prescribed in North America, Western Europe and the rest of the economically developed world (Tyler, 1993, 1994; Blumenthal et al., 1998). Others, however, are being promoted for more general use. For example *Echinacea* (purple cone-flower) is being offered as a general immune system booster (Blumenthal et al., 1998). Other herbal preparations are being marketed for oral use on a regular basis for the prevention of rather unspecific potential conditions or ailments in a prophylactic way. (Why herbal product use is enjoying a significant resurgence in economically developed countries is beyond the scope of this article but for a few different perspectives reference may be made to Astin 1998; Humber and Almeder, 1998, and Krikorian, 1998).

Galenical preparations or herbal mixtures similarly intended for non-specific use(s) were formerly referred to as ‘tonics’ (especially when they were in liquid form and contained some ethanol but indeed even if they were not liquid preparations) (Carson, 1961). However, the expression ‘tonic’ is hardly to be found in the contemporary American herbal medicine literature (see Mowrey, 1993 as an example of an exception). Although it would be wrong to assign the origin of non-specific and restorative tonic preparations to any single culture or geographical region, widespread use seems to be most firmly entrenched in the traditional medical systems of east, northeast, south and southeast Asia.

Contemporary descriptors for what used to be called tonics are suggestive and are more or less tied to what they are supposed to do (Mowrey, 1993). But closer scrutiny of the etymology shows them to be as unrevealing as the word tonic and every bit as non-committal as to their pharmacological action. Expressions like adaptogens, restoratives, harmonizers, adjustives, corrective adjuncts, alteratives, vitalizers, preventives or preventative, oriental adjustive remedies, protectives and even the possibly more scientific-sounding phrase ‘inducers of states of non-specifically increased resistance’ (SNIR) are freely bandied about even though they have until recently been rather vaguely informative at best, even to those professionally versed in clinical pharmacology
and therapeutics. Nevertheless, there are a fair number of traditional medical systems that have incorporated routine use of adjustive preparations or treatments into their health care delivery systems. All of these preparations are used to keep the body in proper tune, so to speak. In the traditional-Hindu medical (Ayurvedic) System of India they are called rasayana (for rejuvenation), in the traditional-Malaysian and Indonesian medical systems they are called jamu, in the fung shui system of China in which one seeks to balance man and nature to create a harmonious environment they are called zi bu or hui fu (meaning tonic and restorative respectively in Mandarin) and in Russian toniziruyushie sredstva (meaning tonic substances). Each of these either connotes or means ‘that which makes new again’, or ‘that which helps restore one’s youthful state of physical and mental health, as well as helps expand a state of happiness’.

We will focus in this review largely on one such ‘non-specific’ agent that is enjoying considerable use namely, Eleutherococcus senticosus (Rupr. & Maxim.) Maxim. This plant is often referred to in the USA as ‘Siberian ginseng’. As we shall see, and contrary to some contentions that Eleuthero-coccus is the ancient source of an ‘adaptogen’ or ‘adaptogenic activity’, its extensive use probably derives only from the mid-1950s and early 1960s. Introduction of the Soviet pharmacopoeial extract of Eleutherococcus into the USA is said to have occurred only in 1971 (cf. Sonnenborn and Hänsel, 1993).

Despite our dissatisfaction with the words ‘adaptogen’ and ‘adaptogenic activity’ (‘why’ will emerge in the course of this review), we have perforce used them here in connection with the non-specific ‘herbal supplement’ for several reasons. The main one is that the word adaptogen developed as a result of clinical pharmacological studies made with Eleutherococcus and hence it is arguably the model source of ‘active’ compounds, whatever that may actually come to mean. And, although we will concentrate on Eleutherococcus, it will be evident that any special concepts that might emerge from our analysis should be broadly applicable to any preparation of, or from, a natural source purported to have adaptogenic qualities (Wagner, 1987, 1990, 1995; Wagner et al., 1994).

2. Eleutherococcus and the adaptogen concept

2.1. ‘Ginsengs’

The family to which Eleutherococcus belongs, the Araliaceae, includes some 84 genera which are native to Asia, the Malay peninsula, Polynesia, Europe, North Africa and the Americas (Wieligorskaya and Takhtajan, 1995). Only the genus Panax and some species of Aralia are herbaceous; the rest are woody vines, shrubs or trees. For many centuries the peoples of China, Korea and Japan have used roots and leaves of Panax ginseng C.A. Meyer (mainly roots however). Following others we will refer to the P. ginseng plant as ‘true ginseng’. (In our view the designation ‘Asian’ or ‘Oriental ginseng’ is less helpful since Eleutherococcus is also ‘Asian’ or ‘Oriental’). True ginseng has been valued for many years as a folk remedy for a wide variety of conditions and ailments as well as a tonic or alterative etc. (Goldstein, 1975; Fulder, 1980a,b, 1993; Baranov, 1982; Leung, 1984; Carlson, 1986; Chong and Oberholzer, 1988; Duke, 1989).

Most readers will readily appreciate that true ginseng is reasonably well known outside east Asian cultures. In fact, because many individuals, scientists and lay people alike, readily recognize the word ginseng, it has fostered a proliferation of terms aiming to capitalize on the positive connotation of anything designated a ‘ginseng’. For instance, the roots and leaves of Withania somnifera L. (Solanaceae) have been used for centuries in Ayurvedic medicine under the name ashwagandha (Atal and Schwarting, 1961; Chemexcil, 1992), and has in some quarters outside India taken on the name of ‘Indian ginseng’. So far as we can determine, Withania has never been referred to as ‘Indian ginseng’ in the scientific literature on Indian medicinal plants (Bhatnagar et al., 1948; Chadha, 1985; Ambasta, 1986; Chemexcil, 1992; Sivarajan and Balachandran, 1994; Rege et al., 1999). Similarly, Pfaffia paniculata Kuntze (Hebanthe paniculata Mart.) (Ama-
ranthaceae, sometimes called ‘suma’) has been referred to outside Brazil as ‘Brazilian ginseng’ (or outside Bolivia as ‘Bolivian ginseng’). It is well-known in Brazil as an aphrodisiac but it is used equally extensively for its alleged anti-diabetic properties (Subiza et al., 1991). Pfaffic acid has been isolated from dry root of the plant and has been reported to be effective as an inhibitor of growth of cultured cancer cells, specifically melanoma (B-16), Hela (S-3) and Lewis lung carcinoma cells at a concentration of 4–6 µg/ml (Takemoto et al., 1983). *P. paniculata* has been marketed in the USA as a component of ‘rainforest ginseng’ along with other allegedly synergistic botanical ingredients like *Lepidium meyenii* (Cruciferae) (sometimes referred to as ‘Peruvian Lepidium’). It will be seen why the designation ‘eleutherokokk’ perhaps best expressed in English simply as ‘eleutherococcus’, or ‘Eleutherococcus’, or even as adopted by some, ‘eleuthero’ (cf. e.g. Tyler, 1994). It will be seen why the designation ‘eleuthero gingseng’ (cf. Veninga, 1976; Zaricor, 1980) is inappropriate. But the main point is that it has never been called ‘Siberian gingseng’ by the Soviets. Indeed, in Russia and other republics of the former USSR, *E. senticosus* has never been viewed or used as an exact substitute for true gingseng, *P. gingseng* (cf. e.g. Baranov, 1982).

In any case, each of these ‘ginsengs’, whether one appreciates their common or invented commercial/marketing names or not, seems to be valuable for a given purpose or in a particular health problem context, and some preparations even combine them. Interestingly, ‘American gingseng’ root is highly regarded in east Asia for its alleged excellent properties. Indeed, oftentimes it is more valued than the root of the true gingseng of the ‘Orient’ and hence has had a long history of being considerably more expensive (Garman, 1898; Kains, 1906; Harding, 1908; Williams, 1957; Graham, 1966; Hardacre, 1968; Proctor and Bailey, 1987; Singer, 1990). It is, however, not generally used as a substitute for the kinds of tonic effect associated with true gingseng. Instead, it is used in their medical system to treat conditions related to ‘heat’ and ‘dryness’, such as fevers and coughs. Leung (1984) points out that ‘American gingseng’ is especially popular with the southern Chinese (e.g. the Cantonese). The action of ‘American gingseng’ root is more normally equated with that of the leaves of true gingseng. Fulder (1980b) on the other hand emphasizes that in the Orient *Eleutherococcus* normally has the reputation of a less effective substitute for true gingseng. Interestingly, there has hardly been any tradition at all of using American gingseng in the USA; indeed it never was a popular home remedy even in the areas where it grows wild (Garman, 1898). Its use is increasing, however, by virtue of the device of being marketed in mixtures of true gingseng in combination with other ‘ginsengs’ (see later in this review). Mention must be made, however, of the recognition of certain medicinal qualities of ‘American gingseng’ root by Native American Indians (Krochmal and Krochmal, 1973; Veninga, 1976)

As might well be expected of a plant that has been known and nominally valued for centuries (at least by some, mainly by those who could
afford it), wild populations of *P. ginseng* have long since been over-exploited. Natural habitat loss has also contributed to the problem of unavailability. Shortages led to inflated prices long ago. To increase availability, sporadic efforts were made in various countries to cultivate true ginseng. It has been cultivated in Korea for some years and is the basis of a substantial domestic and export industry (Fulder, 1993). Cultivation of true ginseng has even been undertaken in a number of locations in the New World, e.g. in Canada especially in the Province of Ontario, near Ottawa, and places such as Wisconsin, USA.

Wild *P. ginseng* was very scarce in the Soviet Far East (eastern Siberia) as well as in Korea and adjacent regions as far back as the 1950s–1960s. Recognizing that it takes a very long time (order of a minimum of 6 years and preferably longer) to cultivate a ‘quality’ true ginseng root efforts were made by Soviet workers to identify an alternative and plentiful plant source that would have similar pharmacological activity and ‘tonic’ properties. It was in this context that scientists in the USSR turned their attention from *P. ginseng* to *E. senticosus* as part of a screening of the Araliaceae family (Brekhman, 1968). As it turns out, this is the only species in the genus which grows in the territories of the former USSR and which has a habit and gross morphology somewhat similar to true ginseng. Both the roots and leaves of *E. senticosus* are reported to be the source of ‘adaptogens’.

*Eleutherococcus* (from the Greek *eleutheros* meaning ‘free’, and *kokkos* meaning ‘pip’ or ‘seed’, more precisely a pyrene in botanical terminology) is a thorny shrub that grows in the Russian Far East, the Amur Region, Primorsky Krai, Sakhalin, and also in Northeast China, Korea and Japan. The thorniness, reflected by the specific epithet *senticosus*, an adjective meaning in Latin ‘full of briers or thorns’, has led to the common names in Russian of ‘thorny *Eleutherococcus*’ (*eleutherokokk koljuchii*), ‘untouchable’ (*nedotroga*), ‘devil’s bush’ (*dyavol’ skii kust*), ‘wild pepper’ (*dikii perets*) or even ‘thorny bearer of free berries’ (*svobodnoj agodnik koljuchii*) (see Mashkovsky, 1988 and Fig. 1). (Incidentally, no one seems to have seriously concerned himself/herself with establishing whether they are in the strictest sense, anatomically, prickles, that is sharp outgrowths from the outer epidermal layer, as in the case of roses which produce prickles not thorns, or whether they are spines, i.e. small thorns or aborted branches or whether they are some other kind of epidermal appendage or emergence formed from both epidermal and subepidermal tissues.) The far less common Russian designation ‘*tajozhnyi koren*’ (taiga root) derives from its association as an understory plant in the northern coniferous evergreen forests of the subarctic region (the so-called taiga bordered on the north by the treeless tundra and on the south by the steppe). (One of the common names of *Eleutherococcus* root in German is Taigawurzel (taiga root, cf. e.g. Bladt et al., 1990); another is Teufelskrallenwurzel (devil’s claw root, not to be confused with the root of *Harpagophytum procumbens* (Burchell) DC of the sesame family, Pedaliaceae, also called Teufelskralle (cf. Sprecher, 1977) but nowadays usually with the qualifier ‘Südafrikanische’ (Teufelskralle) in an attempt to avoid potential (inevitable?) mix-up (Blumenthal et al., 1998). Both are direct translations from the Russian.)

According to some authorities there are about 15 species of *Eleutherococcus*, and all are found in eastern Asia from the Himalayas to Japan. As mentioned above, in the territories of the former USSR there is only one species, *E. senticosus*. On the other hand, some claim there are about twice as many valid species. For instance the New Royal Horticultural Society Dictionary of Gardening (1992) says that there are about 30 species.

*Eleutherococcus* seems to have been first collected some time between 1830 and 1841 by Porphyrii Yevdokimovich Kirilov (1801–1864?). Kirilov was the physician appointed to the 11th Russian Ecclesiastic Mission to Peking (arriving there via Mongolia in 1830 to replace the 10th Mission which had been there since 1820). After years of service and botanizing, Kirilov returned to St Petersburg in 1842. Breitschneider (1898) lists *E. senticosus* and *P. ginseng* among those many plants collected by Kirilov but only described botanically later by CA Meyer, Ruprecht, Regel, Maximowicz (all of the Imperial Botanical Gar-
Fig. 1. Diagrammatic representation of *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Araliaceae). (1), flowering branch; (2), portion of a root cutting used as a propagule showing spiny shoot and lateral buds that have been generated from it; (3), open flower; (4), floral bud; (5), pistil; (6), inflorescence; (7), lateral view of the fruit; (8), view of fruit from above; (9), transverse section of the fruit; (10), seeds. This species is a shrub to about 7 m but it is usually much shorter, usually about to 2 m. The stems are erect, sparingly branched and densely thorny (prickly, spiny?) or even unarmed (form *inermis* Komar). The thorns point backwards. The leaves are three–five foliate, petioles to 12 cm, finely prickly or unarmed. The leaflets are 13–7 cm stalked, elliptic-ovate to oblong. Diagram reproduced from Brekhman (1968) page 11.
den, St. Petersburg). In fact, Carl Ivanovich Maximowicz (1827–1891), the Conservator of the Imperial Botanical Garden in St Petersburg, and later the Head Botanist and supervisor of the herbarium of the Imperial Botanical Garden in St Petersburg, even raised *Eleutherococcus* from seeds that had been sent back to the garden during one of his own trips to the Far East, Manchuria (cf. Bretschneider, 1898 and Fig. 2). Significantly, no mention seems to have been made concerning the use of *Eleutherococcus* as a medicinal plant but this tentative statement merits validation if only for historical purposes.

Only many years later after renewed attention had been paid to *Eleutherococcus* was it claimed by Brekhman (1968) that *Eleutherococcus* had identical or even superior pharmacological effects to those long-attributed to true ginseng (Baranov, 1982). *Eleutherococcus* certainly was and still is easier to find and collect from the wild than its true ginseng relative. During the 1950s and 60s *Eleutherococcus* became the subject of a number of studies from the perspective of its geographical and topographical distribution, as well as the conditions under which it grew (Brekhman, 1968). (Parenthetically, it was also learned that it is considerably easier to cultivate *Eleutherococcus* than true ginseng, see Halstead and Hood, 1984 for a compilation in Latin script of references to some of the Russian literature).

Finally, this seems to be a good place to comment on the botanical nomenclature for *Eleutherococcus*. The valid botanical name is *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. (Soejarto and Farnsworth, 1978). Some literature however, some of it fairly recent, still refers to this same plant under an older, now-relegated-to-synonomy binomial as *Acanthopanax senticosus* with or without the botanical authority (Rupr. et Maxim. ex Maxim.) Harms (see Lui and Staba, 1980; Wagner, 1987, 1990 as examples) or [Rupr. et Maxim.] Harms (see e.g. Bladt et al., 1990). Nevertheless, *Eleutherococcus* and *Acanthopanax* are now generally considered different genera in the Araliaceae. Although *Eleutherococcus* and *Acanthopanax* were merged by Harms in 1898 it has since been established that the oldest validly published name, *Eleutherococcus*, should be retained (Soejarto and Farnsworth, 1978). (Mention will be made later that use of *Eleutherococcus* as a medicinal plant is rather recent; its use under the designation of ‘adaptogen’ is certainly recent. It is beyond the scope of this review article to resolve the question whether this polymorphic genus might in fact have a considerably older usage as an ‘adaptogen’ or for other purposes than its more recent history implies. The use of common names in the older herbal literature (e.g. Chinese) underscores a widespread problem in identification of a given plant and linking it rigorously with a specific use. (A table provided by Bladt et al., 1990 that lists some of the morphological features of some 11 ‘species’ of *Eleutherococcus* emphasizes how trivial the nominally distinguishing features are!) We predict that the satisfactory resolution of this problem will prove to be far more complex than has been presented by Halstead and Hood, 1984).
3. Adaptogens and the Soviet connection

In the late 1940s (ca. 1947) scientists at the Far Eastern Division of the Soviet Academy of Sciences in Vladivostok, Siberia began to study compounds that brought about a state of ‘non-specifically increased resistance’ of an organism (nespetsificheskaya soprotivlyaemost’ organisma) in experimental animals and humans (date cited in Wagner et al., 1994; Wagner, 1995). Later, Grinevich (1990) described searching for prospective medicinal plants by use of a computer. A number of complex remedies from the ‘Cannon of Medicinal Science’ by the great Persian scientist, philosopher and physician Avicenna (Abu Ali ibn Sina), Indian recipes from Ayurvedic medicine, Chinese, Korean and Japanese prescription recipes were compared and contrasted. The criteria for the comparison were the frequency of use of a certain plant in a recipe, the purpose of use of a certain plant in a recipe, and how many plants with the same pharmacological effect were used in a recipe. This search showed that certain plants were used in preparations of the different medicinal systems, even though the countries involved were sometimes geographically quite distant.

According to the late Professor Israel Itskovitch Brekhman (1921–94) (Brekhman, 1968) and Brekhman and Dardymov (1969), it was Dr Nikolai Vasilievich Lazarev (Lazarian in Armenian) (1895–1974), then the leading figure in Soviet pharmacology and toxicology and a developer of a number of new drugs, who first proposed to the scientific and medical community in the mid-1950s that substances which were able to bring about an increased non-specific resistance be called ‘adaptogens’ (adaptogen in Russian, presumably based on the Latin adaptare, to adjust or fit, and ‘gen’ from the Greek genes or born of, or produced by). Incidentally, it is generally regarded as a barbarism to combine Greek and Latin roots in a single word). Terms like ‘revitalizing therapies’ or ‘tonic herbs’ which contain ‘immunopotentiating principles’ or ‘immunomodulatory substances’ (immunopotentsialnyi effekt/immunomoduliruyuzhnie sredstva, respectively, in Russian) are not uncommon in the Soviet literature of the period. Not only are such ‘adaptogenic plants’, with Eleutherococcus as the model, viewed as sources of agents that enhance tolerance to stress, they have even been promulgated as sources of substances that have preventive and anti-cancer activity as well as some other positive activities (Lazarev, 1955; Kupin et al., 1986). It was a slow process however before the idea of an adaptogen now adopted by at least some Western researchers of complementary and alternative therapies, namely that adaptogens are a group of substances reputed to offer varying degrees of protection against internal and external stressors, crept into western consciousness (and vocabulary).

Indeed, an extensive search of the medical, chemical and pharmaceutical literature shows that the word ‘adaptogen’ is really very rarely used. It is not readily retrievable through any of the normal computer searches of scientific literature. In fact, we have not found a definition of any sort in any dictionary, medical or otherwise, English or Russian (Müller, 1989; Akzhigitov et al., 1992; Howlett et al., 1993). ‘Pharmacosanation’ (pharmakosanatsiya) is another term which was used extensively in connection with work or discussions on adaptogens. While it is not a term that is used in the West, in Russian public health and medical science circles it is understood and defined as ‘that part of pharmacology that deals with the effects of biologically active substances in food or medicines that increase stability against various unfavorable effects, that promote prophylaxis and normalize functions...’ (Brekhman, 1980). Viewed from this perspective, there is a special condition characterized by the body’s ability to achieve a state of increased resistance to the damaging action of various substances and agents. Significantly, this state is believed to be attained by gradual (even over a period of months) exposure to the action of an adaptogen against unfavorable factors in the external environment, or even single-dose administration of certain medicinal substances (i.e. an adaptogen or mixture of adaptogens). (For brief summaries of the Russian literature of that period in English see Brekhman, 1980; Halstead and Hood, 1984 or Kamen, 1988 although the latter appears to be more promotional than scientific in intent.)
These various properties of an adaptogen were expanded upon and enumerated in outline form by Brekhman in 1968 and were published in his multidisciplinary publication on *E. senticosus* and *P. ginseng* (Brekhman, 1968). According to Brekhman (1968)

1. The action of an adaptogen should be innocuous and cause minimal disturbance to the normal physiological functions of an organism. It must be absolutely harmless;
2. an adaptogenic agent should not be active only in a specific context or against a particular background. It must have a broad therapeutic spectrum of action;
3. the action of an adaptogen has to be non-specific, that is to say, resistance to a wide variety of action of harmful factors, whether of a physical, chemical or a biological nature, has to increase. In other words, the action of an adaptogen has to be more intense as unfavorable changes occur in an organism;
4. an adaptogen has to have a normalizing or stabilizing action independent of the direction of previous changes.

According to Lazarev and Brekhman, adaptogenic activity can be brought about by quite different substances of very different origin (Brekhman and Dardymov, 1969). Moreover, they probably have different mechanisms of action, but the same pharmacological effect. These many chemical and physical non-specific factors nominally increase the state of resistance of the human body to outside irritants and stresses. Lazarev called this bodily state ‘the state of increased non-specific resistance’ (sostoyanie povyshennoi nespetsificheskoi soprotivlyaemosti in Russian).

From the foregoing, few trained in conventional modern western medicine would be in a position to imagine in very much detail what an adaptogen does or does not do so far as the pharmacologist is concerned, but more importantly, what its specific mechanism of action might be (cf. e.g. Wagner et al., 1994; Rege et al., 1999). In fact, one could easily argue that the concept of an adaptogen as initially proposed by the Soviet workers is imprecise, even vague, and certainly does not lend itself to classification within any broad category of medicinal substance or pharmacological activity. Indeed, one might elect to emphasize and argue that the alleged qualities or properties of an adaptogen as put forward by the inventors of the term apply equally well to a placebo (Frank and Frank, 1991; Harrington, 1997; Shapiro and Shapiro, 1997). We will return to this point later.

Be all this as it may, the writings of Brekhman and his colleagues reflect confidence that they had shown that an adaptogen works for those who are ‘not well’, especially in those cases where there is a need to increase immunity after surgery, or to facilitate convalescence after a serious disease. Equally interesting, however, is that Brekhman et al. purported to have shown that an adaptogen works in healthy individuals as well and that adaptogens help to maintain a healthy state by minimizing the effects of harmful external factors (cf. Brekhman, 1968).

After the term adaptogen was first coined, its use was limited so far as its applicability to substances of plant origin was concerned, to the responses brought about through the activity of *Eleutherococcus* extracts after oral administration. It was later extended to any product of botanical origin that enabled the body to counteract negative effects of ‘stress’, in the broadest sense of that word. Thus, plants such as *Aralia mandshurica* Rupr. & Maxim. and *Aralia cordata* Thunb. (from the family Araliaceae), *Rhaponticum carthamoides* (Willd.) Iljin and *Carlina biebersteinii* Bernh. (Compositae), *Rhodiola rosea* L. from the family Crassulaceae and *Shizandra chinensis* (Turcz.) Baill. from the family Shizandraceae were also advocated by the Soviet scientists as having adaptogenic activities (Brekhman and Dardymov, 1969; Baranov, 1982). In that early period, a wide range of individuals appear to have taken up the use of *Eleutherococcus*, including elite athletes. Soviet-era coaches are reputed to have incorporated regular use of eleutherokokk preparations into their athlete-training protocols (Brekhman, 1968; Fulder, 1980a). Presumably this was because *Eleutherococcus* nominally enhanced ergogenic activity. (Ergogenic is an adjective derived from the Greek word ‘ergo’ for work and ‘gen’, meaning ‘production of’. It is usually
Fig. 3. Photographs of Soviet postage stamps, both 1973. (a) *P. ginseng* (Araliaceae), 2 kopecks. At the top 'Medicinal Plants'. On the left, the common name in Russian, i.e. common ginseng (zhen'shen' obyknovennyi); (b) *Oplopanax elatum* (Araliaceae), 1 kopeck. At the top 'Medicinal Plants'. On the left, the common name in Russian, 'zamanikha vysokya' (literally 'tall enticer'). Personal collection of one of us, ADK.

ginseng and other Araliaceous plants like *Oplopanax elatum* Nak. Indeed, postage stamps to commemorate their use were even issued (see Fig. 3a, b).

A number of studies on the effects of *Eleutherococcus* were carried on human subjects and some of them were done on mice. Subjects were exposed in clinical studies of various designs to stresses such as noise, high altitudes, the running of long distances and being forced to perform some rather complex psychomotor tests. In this research, treatment with *Eleutherococcus* was chosen as the experimental variable and controls comprised true ginseng and placebo (colored water or aqueous alcoholic solutions). Endurance and oxygen uptake were used in some of the trials as fitness indicators. The experiments showed that human subjects who took *Eleutherococcus* extract were enabled to perform tasks most effectively. Those who took ginseng extract performed better than those who took colored water but somewhat worse than the *Eleutherococcus* group (Brekhman, 1968 *passim*). Some of the tests and interpretation of the clinical results pre-dated common use of the word 'adaptogen'. Based on what has been said above, it may be speculated that the authors attributed their findings to what came to be called the 'adaptogenic properties' of *Eleutherococcus* as well as true ginseng without going through extensive or exhaustively detailed reasoning of what might be happening mechanistically.

Nevertheless, in order to appreciate better how the adaptogen concept and the use of adaptogens became so readily integrated into health care practice during that period, or even today might be integrated in the West, some attention will now be devoted to differences between Western-style, conventional medicine and 'Eastern' medicine.

4. Western-style and eastern medicine: a world apart?

It may be argued that contemporary western medicine views illness as due to what might be termed for comparative purposes, and for lack of a better term, a 'malady' at the organ, tissue, cellular, subcellular and molecular level. Treat-
ment is accordingly based on attempting to effect a 're-alignment', or simply physical or psychological treatment of symptoms of a discomfort rather than the cause of it (Clouser et al., 1997; Thagard, 1999). By contrast, traditional Chinese medicine, and similar systems of 'traditional Asian' e.g. east Asian, southeast Asian, south Asian and central Asian (e.g. Tibetan) Medicine, each portray illness or a disease as the result of a dysfunction of the whole system. Here, misalignment is due to the reaction of an organism to 'irritants' of different origin, both internal and external. Therefore, any treatment assigned to a particular disease is normally targeted at the whole system. The contemporary Chinese view of a disease most likely originated from Buddhism, where one of the main postulates is that everything and everybody are interconnected (Hsiao, 1980; Leung, 1984; Huang, 1993; Bivins, 1997).

Stated another way, a disease is the result of external and internal stress(es) on the human body. External stresses may be of a chemical, physical, biological, psychological and/or social origin. Any enumeration of internal stress would necessarily include heredity and, of course, certain diseases or tendencies towards pathological processes can be passed on from generation to generation through the genes (Mulvihill, 1991; Cotran et al., 1994; McKusick, 1998). The age and sex of a person, the nature and type of activity of their nervous system, their so-called constitution, the ability or inability of a given body to cope with stress are also 'internal' and accordingly, all play a significant role as well from the ‘Asian’ perspective (see Weiss, 1987 for example for a good discussion of karma doctrine and how it relates to the Ayurvedic system of India as a determinant of disease etiology). Interestingly, the ancient Greeks, the originators of modern western medicine, similarly viewed a disease as a manifestation of an internal/external imbalance of bodily ‘humors’, and treated it accordingly (Grmek, 1998).

Most would agree that a ‘state of good health’ or what is increasingly being referred to in the USA as ‘wellness’ is a relative state. Many different factors affect this ‘healthy state’ in humans (Clouser et al., 1997). Substances classified as chemical irritants or factors which trigger various pathological pathways fostering manifestation of a disease state may also be interpreted as substances that intrude upon, bother as it were, or have an adverse or positive affect on a given system after contact. Chemical irritants include such things as toxins, venoms, allergens and various compounds, natural or synthetic (see the journals ‘Natural Toxins’ (1992) and Journal of Natural Toxins (1992)).

Microorganisms, viruses, mycoplasmas etc. may be cited as obvious examples of biological or biotic factors, the invasion of which can also cause various diseases (see Table 1 for an admittedly simplistic yet hopefully insightful summary of these concepts). Similarly, modern pathology views a ‘disease’ as the result of cellular injuries and cell death caused by biological, chemical and physical factors (Cotran et al., 1994).

| Table 1 |
|---|---|
| Some factors that are known to be involved in the origin of disease | |
| **Factor** | **Example** |
| Chemical factors | Toxins, venoms, various allergens |
| Biological factors | Microorganisms — bacteria, fungi, mycoplasmas and viruses and products of their metabolism and/or degradation |
| Heredity | Bodily ‘constitution’ and inheritance of the propensity to develop various disease states |
| State of the nervous system | Bodily ‘constitution’ and so-called ‘choleric’, ‘melancholic’, ‘phlegmatic’ and ‘sanguinic’ types of nervous system (see text for further details on these terms) |
| Other(s) | Physical factors such as external injuries which may lead to a disease. The affect of external irritants on an unstable mental system resulting in the triggering of a pathological pathway. Age and sex of a subject which pre-disposes him/her to certain diseases etc. |
It may be appropriate as well to say a few words here on how immunity is variously viewed. Allergens are substances of microbial and fungal etc. origin and their toxins, and any of their degradation products. They also include non-microbial and fungal proteins like those ingested in foods, from air-borne pollen, plant and animal fibers and hairs and any other factor (cf. Table 1). Such allergens bring about an ‘inflammation’; this is the first stage leading to an imbalance in the human immune system (Mulvihill, 1991). The imbalance turns on the body’s cellular machinery to ‘fight foreign intruders’. If, for example, the cellular signaling system is overworked, it will lead to an allergic reaction. Or, if a body is not able to ‘neutralize’ an intrusion, a disease will develop as the result of decreased immunity. In brief then, immunity represents a critical measure of the resistance of an organism to stresses from the external environment, stresses that can result in a diseased state. Immunity could be described equally well as the defensive and adaptive reaction of an organism to stress and insult at the cellular and molecular levels (Sanglier, 1993). Recall that according to the inventors of the adaptogen concept, Lazarev and Brekhman, a substance with adaptogenic activities should be able to help maintain, and when it is necessary, to correct the immune state of an organism.

Irrational nutrition and malnutrition constitute very important stresses, and may be viewed as one category of biochemical irritation to an organism. Irrational nutrition may cause such well-known diseases as obesity, diabetes, atherosclerosis and various types of hypo- or even hypervitaminoses (Cotran et al., 1994). Malnutrition can also cause conditions such as anemias and vitamin deficiencies such as pellagra (deficiency of vitamin B3), rickets (deficiency of vitamin D), beriberi (deficiency of vitamin B1) and scurvy (deficiency of vitamin C) (see e.g. Mason, 1996; Wilson, 1998).

In modern western medicine precise therapies are definitely preferred over agents that purport to have broad or non-specific therapeutic action. Simply stated, there is no need to take medication when it is not required. Moreover, agents that serve to ‘normalize’ and ‘stabilize’ generally are thought to do so by virtue of their ability to minimize or eliminate any potentially adverse psychological component, such as worry. (Hence the widespread use of anxiolytic agents or tranquilizers in many western societies –see e.g. Casey, 1996; Scahill and Skrypeck, 1997). Therefore, only a malfunctioning organ or system needs to be treated or ‘cured’. If in the course of treatment, or afterwards, yet another problem arises, say one that is brought about by the dysfunction of a different system, it too is treated. This is perhaps the single most distinctive feature of the approach taken by practitioners of modern, conventional medicine in dealing with illness (Poynter, 1963 and refs. cited therein). Granted the foregoing, it is interesting to contemplate that an agent that alleviates or eliminates a disease or general ‘condition’ could qualify to be called a ‘non-specific’ or
'cure-all' or even a 'panacea' in western medicine. No less significantly such an agent would also qualify to be called a placebo (Morris, 1992; Shapiro and Shapiro, 1997).

The reader will recall by way of comparison, that in the tradition of holistic medicine historically so assiduously followed in traditional 'Oriental and Asian Medicine', particularly by practitioners of Chinese medicine and its traditional Japanese derivative, 'kampo', the view is that illness is a result of a violation of the balance between an organism and its environment, both internal and external (Bensky and Gamble, 1986; Bensky and Barolet, 1990). That is, when an organism is affected by an irritant or intruding element, the whole organism becomes involved and a disease occurs (Saks, 1997). Again contrast that if you will with the Western viewpoint that maintains that a disease is a pathological state of a cell, tissue, organ, or system and, ultimately the whole organism. Such a pathological state causes a fundamental, i.e. constitutional defect in the affected organ or a systematic dysfunction which perforce originated on a cellular level, indeed at a subcellular and molecular level (Mulvihill, 1991; Cotran et al., 1994).

### 5. Our approach to the adaptogen ‘problem’

Given our interpretation of the facts sketched above, we believed from the outset that it would not be a casual effort to conceptualize what an adaptogen would 'do' medically or how it would ‘work’ from the perspective of contemporary modern western medicine. Therefore, we sought first to examine as wide a range of scientific literature as could be obtained, some rather difficult to locate and access, including many Russian language publications, in an attempt to ferret out as much information on adaptogens as we could. We examined the history, and then we examined the context of the clinical use of adaptogens, and evaluated the experimentation alleging benefits from use. And, proceeding from this essentially historical perspective, we sought to compare and contrast what an adaptogen ‘really’ is, does, or is expected to do for a user, with more conventional, ‘specific’ medicines. This was all done against a range of terms, some familiar, others not-so-familiar, used throughout the history of medicine and pharmacy in the hope that we might reach a better understanding of an adaptogen’s ‘nature’. We also attempted to compile as complete a list as possible of the secondary chemical constituents of *Eleutherococcus* which have been reported to have pharmacological activity. Finally, we tried to compare systematically and from several perspectives the chemical composition of *Eleutherococcus* with that of true ginseng to see if any common features could be disclosed. This, then, is what this paper is all about. And to prepare the reader for what follows, we categorically state here that despite any shortcomings of the definition of adaptogen, or even the concept of an adaptogen, and there are many, there is considerable evidence that supports the view that *E. senticosus* is a potentially important source of clinically active substances. We provide and develop the evidence and present rational arguments for our stance in the following sections.

### 6. Results

#### 6.1. Chemical composition of *Eleutherococcus*

Not surprisingly, it was the same workers who carried out clinical research on *Eleutherococcus* and adaptogens, namely Brekhman et al., who were the first to carry out phytochemical studies on the plant. Working in the late 1950s and 60s in Siberia, chemical investigations were made alongside the pharmacological work (Brekhman, 1968, 1976). Somewhat later, in the 1970s and 80s, the study of the chemical composition of *Eleutherococcus* was taken up by several other groups, namely in Germany, Japan and China (see Table 2). It may be stated, incidentally, that whereas phytochemical studies on *Eleutherococcus* seemed at the outset to have attracted a fair amount of interest, this interest seems to have latterly waned considerably. A recent search of the literature through the NAPRALERT (NAtural PRoduct ALERT) database carried out to supplement our
Table 2
Some chemical components of *Eleutherococcus senticosus* and their reported pharmacological effects

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Botanical source (family based on Wielgorska and Takhtajan, 1995) or other/commercial source</th>
<th>Attributed pharmacological activity</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syringin*</td>
<td><em>Acanthopanax senticosus</em> (Araliaceae), <em>(E. senticosus)</em></td>
<td>Protectant against damage from radiation&lt;sup&gt;a,b&lt;/sup&gt;. Fewer deaths occurred in mice after X-ray irradiation (400 rads); decreased leucopenia, improved white blood cell count and thrombocyte level in human workers after exposure to unspecified radioactive substances (Ruijun et al., 1990)</td>
<td><img src="image1.png" alt="Syringin Structure" /></td>
</tr>
<tr>
<td>Syringin</td>
<td>Syringin from <em>Tinospora cordifolia</em> (Menispermaceae); natural product chemistry division of regional research laboratory, Jammu Tawi, India</td>
<td>Immunopotentiating/immunostimulatory effect&lt;sup&gt;c&lt;/sup&gt;. Inhibited immunohaemolysis of antibody-coated sheep erythrocytes by guinea pig serum (Kapil and Sharma, 1997)</td>
<td>See above structure</td>
</tr>
<tr>
<td>Sesamin</td>
<td>Not specified</td>
<td>Hypocholesterolemic effect&lt;sup&gt;d&lt;/sup&gt;. Induced hypocholesterolemia (especially low-density lipoproteins and cholesterol, which are risk factors for human atherosclerosis) (Hirata et al., 1996)</td>
<td><img src="image2.png" alt="Sesamin Structure" /></td>
</tr>
<tr>
<td>Sesamin</td>
<td>Mixture of sesamin and episesamin provided by Suntory, Osaka, Japan</td>
<td>Anti-cancer effect&lt;sup&gt;e&lt;/sup&gt;. Showed 36% reduction in 7,12-dimethylbenz[a]anthracene-induced mammary cancer in female rats at 12 weeks after uptake (Hirose et al., 1992)</td>
<td>See above structure</td>
</tr>
<tr>
<td>Sesamin</td>
<td>Crystalline mixture of sesamin and episesamin (1:1), from Takemoto Oil and Fat, Gamagori, Japan</td>
<td>Immunostimulatory effect&lt;sup&gt;c&lt;/sup&gt;. Decreased liver enlargement caused by ethanol intake; increased the concentration of IgG [γ-immunoglobulin] (Nonaka et al., 1997)</td>
<td>See above structure</td>
</tr>
<tr>
<td>Chemical constituent</td>
<td>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</td>
<td>Attributed pharmacological activity</td>
<td>Chemical structure</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>Sesamin</td>
<td>Mixture of sesamin and episesamin prepared and purified from refined sesame oil by authors</td>
<td>Improved impaired liver function in rodents caused by 1% ethanol or carbon tetrachloride (100 mg/kg) (^7) (Akitomo et al., 1993).</td>
<td>See above structure</td>
</tr>
<tr>
<td>(\beta)-sitosterol (many synonyms, e.g. (\beta)-sitosterol-3-(\beta)-D-glucopyranoside)</td>
<td>Sigma Chemical, St Louis, MO, USA</td>
<td>Anti-cancer effect(^6). Inhibited growth of human colon cancer cells (HT-29) by activating sphingomyelin cycle (Awad et al., 1998)</td>
<td></td>
</tr>
<tr>
<td>(\beta)-sitosterol</td>
<td>Cyperus rotundus (Cyperaceae). Unclear whether isolated by authors.</td>
<td>Anti-inflammatory and anti-pyretic effect(^5) similar to that of acetylsalicylic acid (Gupta et al., 1980).</td>
<td>See above structure</td>
</tr>
<tr>
<td>Sitosterol</td>
<td>Not specified</td>
<td>Hypcholesteremic effect(^4). Administered doses reduced cholesterol absorption in man (Heinemann et al., 1993).</td>
<td>See above structure</td>
</tr>
<tr>
<td>(\beta)-sitosterol and (\beta)-sitosterol-3-(\beta)-D-glucoside</td>
<td>(\beta)-sitosterol was obtained from ‘Dr Esteve’ Laboratory; (\beta)-sitosterol and (\beta)-sitosterol-3-beta-D-glucoside was prepared by semi-synthetic method by the authors</td>
<td>Anti-hyperglycemic effect; insulin-reducing effects mostly due to (\beta)-sitosterol(^8). Increased fasting plasma insulin level; decreased fasting glycemia after oral administration. Both compounds increased glucose-induced insulin secretion. Overall improvement in results in oral glucose tolerance test (Ivorra et al., 1988)</td>
<td>See above structure</td>
</tr>
<tr>
<td>(\beta)-sitosteryl-(\beta)-D-gluco-pyranoside</td>
<td>Not specified</td>
<td>Hypcholesterolic effect(^d). Sitosterol glucoside was able to bind to low density lipoproteins (LDL) and decreased vascular permeability; also showed hemostatic effect (Sugiyama and Seki, 1991)</td>
<td>See above structure</td>
</tr>
</tbody>
</table>
Table 2 (Continued)

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</th>
<th>Attributed pharmacological activity</th>
<th>Chemical structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hedera saponin B</td>
<td><em>Hedera helix</em> L. (Araliaceae)</td>
<td>Anti-leishmanicidic effect but not confirmed (Majester-Savornin et al., 1991)</td>
<td><img src="image" alt="Hedera saponin B" /></td>
</tr>
<tr>
<td>Isofraxidin</td>
<td><em>Micandra elata</em> (Euphorbiaceae)</td>
<td>Anti-cancer activity*, Cytotoxic in lymphocytic leukemia in mice (Borris et al., 1980)</td>
<td><img src="image" alt="Isofraxidin" /></td>
</tr>
<tr>
<td>Isofraxidin</td>
<td><em>Artemisia abrotanum</em> L. (Asteraceae)</td>
<td>Choleretic effect when administered at 25 mg/kg (Danielak et al., 1973)</td>
<td><img src="image" alt="Isofraxidin" /></td>
</tr>
<tr>
<td>Chemical constituent</td>
<td>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</td>
<td>Attributed pharmacological activity</td>
<td>Chemical structure</td>
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<td>-----------------------</td>
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</tr>
<tr>
<td>Caffeic acid</td>
<td>Sigma Chemical, St. Louis, MO, USA (Catalogue # C-0625)</td>
<td>Anti-oxidative effect*. Anti-xanthine oxidase activity. Anti-tumor, anti-gout, anti-hepatitis activity (Chan et al., 1995)</td>
<td>![Caffeic acid structure]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>ICN Biomedical Irvine, CA, USA</td>
<td>Anti-oxidative effect*. Caffeic acid and other phenolic compounds from green tea showed reduction of nitric oxide production in C6 astrocyte cells (Soliman and Mazzio, 1998)</td>
<td>![Caffeic acid structure]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Not specified</td>
<td>Antibacterial effect†. Inhibited growth and production of aflatoxin by <em>Aspergillus parasiticus</em> (Aiz et al., 1998)</td>
<td>![Caffeic acid structure]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Not specified. Apparently they quantified caffeic acid along with other phenolic compounds in a given ingested food source</td>
<td>Anti-oxidative and anti-cancer effects when administered at 206 mg/d*†† (Radtke et al., 1998)</td>
<td>![Caffeic acid structure]</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>Not specified</td>
<td>Anti-oxidant and anti-cancerogenic effects*††. Inhibited tumor promotion in vivo and vitro with murine peritoneal macrophages when they treated with tumor promoters and produced superoxide anions (Kaul and Khanduja, 1998)</td>
<td>![Caffeic acid structure]</td>
</tr>
<tr>
<td>Coniferyl aldehyde</td>
<td>Clove buds from Takasago Perfumery. The compound was obtained from clove bud oil in the authors’ laboratory</td>
<td>Anti-oxidative effect†. Anti-oxidant against skin damage induced by UV light-derived hydroxyl radicals (Taira et al., 1992)</td>
<td>![Coniferyl aldehyde structure]</td>
</tr>
<tr>
<td>Chemical constituent</td>
<td>Botanical source (family based on Wielgorska and Takhtajan, 1995) or other/commercial source</td>
<td>Attributed pharmacological activity</td>
<td>Chemical structure</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td>Caffeic acid ethyl ester</td>
<td>Prepared by authors</td>
<td>Anti-oxidative effect*. Protectant against single stranded DNA breaks in Chinese hamster V79 cells caused by hydrogen peroxide. Overall protection against cell damage (Nakayama et al., 1996)</td>
<td></td>
</tr>
</tbody>
</table>

* Given as the active component; however it is unclear from the context of the work whether or not the compound was tested alone.

* Anti-oxidant.

* Active against radioactivity damage.

* Immunostimulant.

* Hypo-cholesteroletic.

* Anti-cancer agent.

* Improved liver damaged caused by alcohol (anticirrhotic).

* Anti-inflammatory.

* Hypoglycemic.

* Choleretic (or cholagogic).

* Antibacterial.
An intrinsic feature of *Eleutherococcus* chemistry, which is more or less common to all phytochemistry is that the relevant chemical nomenclature is not uniform. Even today, there is no universally adopted system or formally accepted protocol for giving common or trivial names to similar or even identical chemical compounds from various plants. Utilizing a botanical generic name followed by a traditionally accepted suffix to refer to various classes of components has only served to generate confusion in our view, although all appreciate that it is arguably intrinsically necessary to give a crude mixture or a fraction some sort of name so as to allow preliminary pharmacological and clinical studies to go forward. After all, jargon is the currency that facilitates scientific communication. For instance, we see true ginseng (*P. ginseng*) saponin compounds referred to with equal frequency as panaxosides or ginsenosides Ra, Rb, Rc etc. (the various ‘Rfs’ deriving historically from the relative positions of spots (relative to the front’ = Rf) on plates after separation and disclosure via thin layer chromatography, see e.g. Lui and Staba, 1980). Thus, Rg has been sometimes treated or referred to as a single compound from true ginseng. However, the designation Rg has also been used to designate total saponin content in ginseng. *E. senticosus* provides an equally good example of nomenclature and identity problems. According to Brekhman (1968) and Dardymov (1976), eleutheroside A is identical to the sapogenin dauko sterol (originally identified from carrot, *Daucus*), which is 3-O-β-sitosterin glucoside, and eleutheroside B is syringin (a lignan isolated initially from lilac, *Syringa*), which is the 4-β glucoside of sinapyl alcohol. However, some, for instance Ruijun et al. (1990), have apparently preferred to refer to eleutheroside A as the lignan syringin. Undoubtedly some of the ‘chemical synonymy’ is due to precipitous publication of names and structures without adequate study of the literature. In other words, the chemistry problem derives largely from the fact that pharmacological or clinical effects of these phytochemicals are often defined earlier than the chemical composition is rigorously determined and verified. Mention was just made that eleutheroside A is identical to daucosterol. Likewise eleutheroside B4 is identical to sesamin; eleutheroside B1 is isofraxidin which is more accurately known as isofraxidin-7-O-β-L-glucoside, or syringaresinol; eleutheroside E is (−)-syringaresinol-4,4’-O-β-D-diglucoside, also identical to acanthoside D; eleutheroside E4 is (−)-syringaresinol-0-β-D-monoglucoside and so on (cf. e.g. Bladt et al., 1990).

It is perhaps understandable that some sort of name is deemed useful to convey a preliminary indication of what the active components might be provided the compounds in question are novel. One is less sympathetic to hasty publication when the structures are rather well known. The practice of giving a name to compounds in a way that implies that they are somehow distinctive to the genus or species in question is unfortunate from several perspectives, not the least of which is that many plants, including rather unrelated genera, synthesize similar or identical secondary compounds (cf. e.g. Romeo et al., 1996). It is regrettable that the Soviet phytochemists classified from the outset a wide range of very different substances from *Eleutherococcus* under the heading ‘eleutherosides’, even though some of them ought to have been recognized as fairly prevalent in the plant kingdom. In the case of true ginseng at least, ‘ginsenosides’ typically belong to the same group of substances. In an attempt to bring some kind of order to this situation Wagner (1980) has since urged that the major eleutherosides be divided into two classes:

1. the triterpenoidal saponins which are glycosides of oleanolic acid (historically referred to as eleutherosides I, K, L, and M);
2. the phenylpropane derivatives (e.g. eleutherosides B, B1, D and E) most of which are glycosylated (Sonnenborn and Hänsel, 1993).
The problem is that it is very difficult to retroactively make ‘corrections’. We have avoided the problem in large measure by attempting to adhere to the known chemistry but as we shall see, this does not always work.

Table 2 lists a number of secondary compounds from *Eleutherococcus* reported by various authors. The substances are not only found in *E. senticosus* but they occur in a diverse range of plants belonging to various families. The table also shows that compounds from *Eleutherococcus* fall into different chemical groups with various reputed pharmacological effects. Some of the compounds bring about rather different responses at the clinical level. Most of them, like betulinic and caffeic acid, sesamin and syringin, vitamin E and β-carotene, sitosterol and daucosterin, have been shown to exert anti-oxidative and/or anti-cancer effect(s) on one or more cultured cell lines.

The literature shows that the constituents listed have been tested in widely accepted medical tests and assays, and that their alleged effects have generally been more or less validated by different authors. Even though much of the pharmacological data listed in Table 2 derives from investigations using compounds or substances obtained from plants other than *E. senticosus*, we emphasize that each of them occurs in *Eleutherococcus*. Thus, *Eleutherococcus* and the other plant sources cited share a common chemistry, and hence we presume a potential common pharmacology, so far as one or other constituent is concerned. Unfortunately, there is virtually no data on the quantities of a given class of secondary compounds that a given species of the group we are interested in might produce. These compounds include but are not limited to phenylpropanoids (e.g. syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), lignans (e.g. sesamin, syringoresinol and its glucoside), saponins (e.g. daucosterol, β-sitosterol, hederasaponin B), coumarins (e.g. isofraxidin and its glucoside) and vitamins like vitamin E, and provitamins like β-carotene.

It has been suggested (but not proven obviously) that lignans, like many other secondary products, are evolutionarily-derived by elaboration of the phenylpropanoid pathway for a plant’s own ‘benefits’, as its own immunoprotection and protection, as it were, from harmful free radicals (see Lewis and Davin, 1994). Significantly, a number of lignans have been reported to have anti-tumor activity in animals and humans. For example, the lignan podophyllotoxin and related compounds have been studied for their anti-tumor activities and this has led to the design of new anti-cancer drugs (Ayres and Loike, 1990; Daley et al., 1998; Donelli et al., 1998; Subrahmanyam et al., 1998). However, podophyllotoxin is most widely used today to treat venereal warts, condyloma acuminata (White et al., 1997; Tyring et al., 1998; Jablonska, 1998).

Eleutherococcus not only synthesizes lignans such as syringin, syringoresinol and sesamin, but also makes and accumulates lignan precursors such as cell wall-bound hydroxycinnamic acid-caffeic acid and other intermediate compounds of lignan synthesis such as coniferylaldehyde. These precursors have been shown to have significant anti-cancer activity in various laboratory assays as well (Taira et al., 1992; Chan et al., 1995; Aziz et al., 1998; Kaul and Khanduja, 1998; Radtke et al., 1998; Soliman and Mazzio, 1998).

6.2. *Eleutherococcus versus true ginseng* (*P. ginseng*)

During our study of the literature on adaptogens, it became apparent that it would be instructive to compare and contrast *Eleutherococcus* and true ginseng from as many vantagepoints as possible, especially their phytochemistry and pharmacology. As mentioned above, the use of true ginseng in herbal preparations as an ‘adaptogen’ long before the term was coined goes back many centuries (cf. e.g. Lee, 1956; Chen, 1973; Goldstein, 1975; Fulder, 1980b; Leung, 1984; Fulder, 1993).

Table 3 lists to the extent possible the secondary chemical composition of *P. ginseng*. Unlike the summary data presented in Table 2 on *Eleutherococcus*, the pharmacological information in Table 3 derives exclusively from publications relating to true ginseng. It has been claimed (Fulder, 1980b; Zaricor, 1980) and reported later with experimentally supported data by Konoshima (1996) that the active compounds of red ginseng...
Table 3
Some chemical components of *P. ginseng* and their reported pharmacological effects

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</th>
<th>Attributed pharmacological activity</th>
<th>Chemical structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenosides Rb₁ and Rg₁</td>
<td>Not specified</td>
<td>Anti-oxidative effect; prevention of senile decline, Protected neuronal cells from overproduction of nitric acid (Kim et al., 1998d)</td>
<td>![Chemical structures]</td>
</tr>
</tbody>
</table>
Table 3 (Continued)

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</th>
<th>Attributed pharmacological activity</th>
<th>Chemical structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginsenosides Rb₁ and Rg₁</td>
<td>Rb₁ and Rg₁ from Korea Ginseng and Tobacco Institute, Taejon, Korea</td>
<td>Modulated methamphetamine-induced behavior (hyperactivity and conditioned place preference) in pre- and post-synaptic dopaminergic receptors (Kim et al., 1998b)</td>
<td><img src="image1" alt="Chemical structures" /></td>
</tr>
<tr>
<td>Ginsenoside Rg₁</td>
<td>P. ginseng (Araliaceae) not clear whether unpurified saponins were isolated by authors or provided by someone else</td>
<td>Anti-impotence effect. Increased incidence of copulatory behavior in mice when administered at 2.5, 5, 10 mg/kg (Yoshimura et al., 1998)</td>
<td><img src="image2" alt="Chemical structures" /></td>
</tr>
<tr>
<td>Ginsenoside Rg₁</td>
<td>Rg₁ from Korea Ginseng and Tobacco Institute, Taejon, Korea</td>
<td>Nominal preventive effect and agent for treatment of adverse effects of morphine. Inhibited catecholamine secretion at presynaptic sites (Kim et al., 1998a)</td>
<td><img src="image3" alt="Chemical structures" /></td>
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</tbody>
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See above for structure of Rb₁. See above for structure of Rg₁.
Table 3 (Continued)

<table>
<thead>
<tr>
<th>Chemical constituent</th>
<th>Botanical source (family based on Wielgorskaya and Takhtajan, 1995) or other/commercial source</th>
<th>Attributed pharmacological activity</th>
<th>Chemical structures</th>
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<tr>
<td>Ginsenoside Rd</td>
<td>Rd was isolated and prepared by authors from Korean ginseng radix (<em>P. ginseng</em>)</td>
<td>Anti-oxidative effect: Prevented free-oxygen radicals from attacking cell membranes (Yokozawa et al., 1998).</td>
<td>![Chemical structure of Ginsenoside Rd]</td>
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<tr>
<td>Ginsenoside Rf</td>
<td>Not specified</td>
<td>Inhibited tonic pain in mice&lt;sup&gt;d&lt;/sup&gt; (Mogil et al., 1998)</td>
<td><img src="image1" alt="Chemical structure of Ginsenoside Rf" /></td>
</tr>
<tr>
<td>Ginsenoside Rg&lt;sub&gt;3&lt;/sub&gt;</td>
<td><em>P. ginseng</em> (Araliaceae)</td>
<td>Inhibited secretion of catecholamine from acetylcholine-stimulated cells (Tachikawa et al., 1997)</td>
<td><img src="image2" alt="Chemical structure of Ginsenoside Rg&lt;sub&gt;3&lt;/sub&gt;" /></td>
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<tr>
<td>Chemical constituent</td>
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<tr>
<td>20(S)- and 20(R)- ginsenosides Rg₅⁺</td>
<td>Red ginseng was supplied from the Korea Ginseng and Tobacco Research Institute, Tajeon, Korea</td>
<td>Anti-inflammatory effect. The ginsenosides showed receptor binding antagonist activity against Platelet Activating Factor (a lipoprotein) (Jung et al., 1998)</td>
<td>![Chemical structure of 20(S)- and 20(R)- ginsenosides Rg₅⁺]</td>
</tr>
<tr>
<td>PPD (protopanaxadiol the aglycon of Rh₂)</td>
<td>Purified Rh₂ and its aglycon provided by Dr I Kitagawa, Osaka University, Osaka, Japan</td>
<td>Anti-cancer effect. PPD &gt; Rh₂ inhibited growth of B16 melanoma cells at the G1 phase of cell cycle. PPD-treated cells needed shorter time than Rh₂-treated cells. Both Rh₂ and PPD increased melanin production and intracellular adhesion (Ota et al., 1991)</td>
<td>![Chemical structure of PPD (protopanaxadiol the aglycon of Rh₂)]</td>
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See below for Rh₂
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<tr>
<td>Ginsenoside Rh$_2$</td>
<td>Ginseng and Tobacco Research Institute, Taejon, Korea and Seikanshou Korean Company, Japan</td>
<td>Rh$_2$ inhibited growth of human ovarian cancer cell lines at 10–60 μM in vitro (best results were observed when Rh$<em>2$ was tested in combination with cis-diaminedichloroplatinum (II), CDDP; stimulated programmed cell death (apoptosis) at a dose around IC$</em>{50}$ (Nakata et al., 1998)</td>
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<td>Ginsenoside Rh$_2$</td>
<td>Rh$_2$ was obtained from Dr Shin Il Kim, Korea Ginseng and Tobacco Research Institute, Taejon, Korea</td>
<td>Anti-cancer effect$^a$. Promoted apoptosis of human hepatoma SK-HEP-1 cells (Park et al., 1997)</td>
<td>See above for structure</td>
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<tr>
<td>Ginsenoside Rb$_1$, Rb$_4$$^*, Rb_3$, Rc, Rg$_1$, Rg$_2$, Re, Rh$_6$</td>
<td>Not specified</td>
<td>Anti-oxidative effect$^a$. Decreased the level of free radicals induced by xanthine/xanthine oxidase (Guogan and Yan, 1997)</td>
<td>See above for structures of Rb$_1$ and Rg$_1$.</td>
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![Chemical structures diagram](image)
Table 3 (Continued)

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![Chemical structure image]
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</tr>
</thead>
<tbody>
<tr>
<td>Ginsenosides Rf, Rc, Rg, Rb1</td>
<td>Korea Ginseng and Tobacco Research Institute, Taejon, Korea</td>
<td>Anti-stress effect. Order of activity was: Rf &gt; Rc &gt; Rg &gt; Rb1. Ginsenosides inhibited calcium (Ca2+) current and cell membrane capacitance in rat adrenal chromaffin cells. Regenerated catecholamine secretion in adrenal chromaffin cells (Kim et al., 1998c)</td>
<td>See above for structures</td>
</tr>
</tbody>
</table>

* Name of the structure as given by Jung et al., 1998.
** Chemical structure unavailable.
* Anti-oxidant.
* Anti-impotence effect.
* Prevented and treated adverse effects of morphine.
* Tonic pain-killer.
* Anti-inflammatory.
* Anti-cancer agent.
are more potent than those from white ginseng. (No direct comparison in terms of chemistry or amounts of putatively active substances seems to have been done of the components of true ginseng prior to and after processing.)

Although *E. senticosus* and *P. ginseng* are both from the Araliaceae, their chemical composition is very different (cf. Tables 2 and 3). Brekhman (1968) noted and emphasized this long ago. The secondary compounds of *Eleutherococcus* are from different chemical groups such as lignans, saponins, coumarins, vitamins, various sugars and various other even today-unidentified/unnamed compounds. On the other hand, most of the secondary compounds from *Panax* are saponins which, differ from each other by one or a few chemical groups at most. Not only are the chemical compositions of true ginseng and *Eleutherococcus* different, there are differences in their effects on various cultured cell lines (and one might pardonably extrapolate this observation to the human body). From the perspective of their respective chemistries, there seems little basis to use one species as a substitute for another as was initially reported in the early publications of Brekhman and co-workers (see Brekhman 1968, 1976 for comprehensive background bibliographies). True, each of the plants contains anti-oxidants and/or potential anti-cancer compounds, but they differ in a number of other constituents, the pharmacological effects of which do not overlap. True ginseng compounds have been found to have anti-stress activity, to work against male erectile dysfunction (impotence), to kill tonic pain, and even to prevent and treat the adverse effects of morphine (cf. Table 2). The compounds isolated from *Eleutherococcus* include phenylpropanoids (e.g. syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), lignans (e.g. sesamin, syringoresinol and its glucoside), saponins (e.g. daucosterol, β-sitosterol, hederasaponin B), coumarins (e.g. isofraxidin and its glucoside), the triterpene betulinic acid, and vitamins (e.g. vitamin E) and provitamins (provitamin A, i.e. β-carotene).

It will be apparent from Table 3 that most of the reportedly active constituents of true ginseng are not only saponins, but they are rather closely related saponins. They share the same cyclopentanoperhydrophenanthrene ring structure, and differ from each other only in their side chains, and rather minimally at that. Even so, the effects of these saponins on various cultured human cell lines are said to be very different. Assertions in the writings of Duke (1989) and Fulder (1980b) and a number of other authors that true ginseng and *Eleutherococcus* have similar activities and thus may be used interchangeably are not supported by any literature citations. Obviously, just because two plants are from the same family and ‘look alike’ does not mean that they can logically be used as a substitute of one another. The supposed interchangeability between true ginseng and *Eleutherococcus* is a bit reminiscent of those times when the ‘doctrine of signatures’ or ‘similitudes’ was used as a guide to nominal pharmacological activity (Court, 1985).

As can be learned from Table 2 each *Eleutherococcus* compound has been shown to have an anti-oxidative effect on more than one tissue or cell type. For example, the lignan syringin displayed a protective effect against X-ray radiation on human leukocytes and thrombocytes. Also, syringin inhibited haemolysis of antibody-coated sheep erythrocytes in guinea pig. The lignan sesamin showed an ability to decrease low density lipoproteins in humans as well as an ability to inhibit activity of cancer promoting 7, 12-dimethylbenz[a]anthracene in mammary cancers in female rats. It also decreased enlarged liver caused by excessive alcohol intake and was able to increase immunoglobulin g levels. The compound β-sitosterol has been shown to have anti-oxidative potential as an inhibitor of human colon cancer (ht-29), and as an anti-inflammatory and anti-pyretic agent. It also reduced dietary cholesterol absorption in humans and was able to reduce levels of insulin. The coumarin isofraxidin, reported in Table 2, showed cytotoxicity in mice lymphocytic leukemia and stimulated bile as well (i.e. it acted as a choleretic). Caffeic acid has been shown to inhibit xanthine oxidase activity and nitric oxide production, and inhibits the toxic action of aflatoxin from *Aspergillus parasiticus*. Caffeic acid ethyl ester was shown to be a protectant against single stranded DNA breaks in Chi-
nese hamster (v79 cells). Coniferyl aldehyde also showed activity as a protectant against DNA breaks caused by UV-light.

In dramatic contrast to *Eleutherococcus* compounds, the active compounds isolated from true ginseng and listed in Table 3 are all saponins but with rather different pharmacological effects. Most of the active constituents of true ginseng were tested in combinations of two or more, which makes it difficult to establish whether or not each compound has its own pharmacological activity, or only reflects synergistic effects of certain combinations. Rb₁ and Rg₃ have been shown to decrease nitric acid production and have nominally prevented senile decline by the measure of dehydrogenase activity (neuronal viability) and cell integrity by spectrophotometric measurement of LDH (lactic dehydrogenase) in culture medium of neuronal cells. Whereas, Rb₁ and Rg₁ inhibited amphetamine (methamphetamine)-induced hyperactivity and conditioned place preference in mice, Rg₁ alone has been reported to counteract impotence and stimulated copulatory behavior in mice. It also to act as a modulator or even an antagonist of the adverse effects of morphine. By itself, Rg₃ has been shown to inhibit catecholamine secretion by acetylcholine-stimulated cells. Combinations of the 20(S)- and 20(R)-ginsenosides of Rg₃ have been reported as having anti-inflammatory effects by acting as receptor-binding antagonists against platelet activating factor. Rd has been reported as a protector of cell membranes against free oxygen attacks. On the other hand, Rf has been reported as inhibiting tonic pain in mice due to some unexplained process(es). Rh₂ and its aglycon PPD (i.e. protopanaxadiol) have been reported as anti-cancer agents. In particular, Rh₂ was tested on different cancer cell lines and exhibited an anti-cancer effect by selectively promoting programmed cell death — apoptosis — of cancer cells. The combination of seven metabolites synthesized in ginseng, Rb₁, Rb₄, Rb₃, Rc, Rg₁, Rg₂, Re, Rh₁, were shown to decrease free radicals induced by xanthine and xanthine oxidase. A qualitative study of five active compounds from true ginseng, Rf, Rc, Re, Rg₁, Rb₁, showed an anti-stress effect on chromaffin-staining adrenal cells.

7. Discussion

7.1. The comparative chemistry and pharmacology of *eleutherococcus* and *true ginseng*

7.1.1. *Eleutherococcus*

Even though we recognized that the historical definition of an adaptogen would be difficult to defend on the basis of rigorous concepts as recognized in western-style medicine, we decided to give the proponents of the term adaptogen the benefit of the doubt. Therefore, we took the approach in our critical analysis of the literature that it might be profitable to seek some hitherto unappreciated or underappreciated avenue that might explain the reported value of adaptogens. Our detailed examination of the relevant literature summarized in Tables 3 and 4 has shown that *E. senticosus* undoubtedly contains constituents that show pharmacological effects when tested in medically accepted tests and assays. Admittedly not all the pharmacological data presented in Table 2, or even most of it, derives from work with *Eleutherococcus*, but there are a number of kinds of compounds in *E. senticosus* that have been studied for their pharmacological action using preparations from other plants, many of them botanically unrelated, that share a common chemistry so far as one or other constituent is concerned. These compounds include phenylpropanoids (e.g. syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), lignans (e.g. sesamin, syringoresinol and its glucoside), saponins (e.g. daucosterol, β-sitosterol, hederasaponin B), coumarins (e.g. isofraxidin and its glucoside), vitamins (e.g. vitamin E) and provitamins (provitamin A, i.e. β-carotene). These molecules have been shown to have a wide range of pharmacological activities, and an associated literature much broader than can be presented here for reasons of space. Six secondary compounds found in *Eleutherococcus* have been shown to have various levels of activity as antioxidants (syringin, caffeic acid, caffeic acid ethyl aldehyde, coniferyl aldehyde), four have been shown to have anti-cancer effects (sesamin, β-sitosterol, isofraxidin), three have been shown to have hypcholesterolemic activities (sesamin, sitosterol, β-sitosterol and β-sitosterol 3-D-gluc-
coside), two have been shown to have immunostimulatory activity (sesamin, syringin), one has choleric activity (isofraxidin) and one had the ability to decrease moderate insulin levels (β-sitosterol and its glucoside), and at least one (syringin) has shown activity as a radioprotectant, one showed anti-inflammatory and antipyretic activities (β-sitosterol) and yet another has shown activity as an antibacterial agent (caffeic acid).

The research cited here using these compounds was carried out using different cell lines as well as on small animals and human subjects. Interestingly, some of them had more than one effect, and although some of the chemical constituents of Eleutherococcus are from different chemical classes, some of them show similar pharmacological effects. And, despite the fact that ginseng and Eleutherococcus are both in the same family, it is worth re-emphasizing that the secondary compounds of true ginseng are mostly saponins whereas those of Eleutherococcus are lignans (syringin, sesamin), saponins (daucosterol, β-sitosterol, hederasaponin B), phenylpropanoids (syringin, caffeic acid, sinapyl alcohol, coniferyl aldehyde), pro-vitamin and vitamin (β-carotene, vitamin E) and coumarins (isofraxidin and its glucoside).

It is well known that reactive oxygen species can damage different systems and cells within these systems and, therefore, may precipitate or cause a number of diseases ranging from such diseases as atherosclerosis, brain and heart ischemia, radiation damage and cancer, to infection and shock and infection (see Gutteridge and Halliwell, 1994; Shahidi, 1997 and refs. cited therein). As shown in Table 2, all active compounds isolated from Eleutherococcus, but reported from different botanical sources, had various anti-oxidative effects. (see Sies, 1994 and refs. cited therein as to how anti-oxidant action can be manifested at the clinical level.)

In addition to studies of chemical composition of Eleutherococcus plants, cell culture studies by Gui et al. (1991) on multiplication of Eleutherococcus via somatic embryogenesis have shown that embryoids developed normally. However, there was no mention of the yield of plant secondary metabolites that might be involved in their production. It would be very interesting indeed if the saponins could be produced in vitro under aseptic culture conditions.

### 7.2. True ginseng

Once again we emphasize that although Eleutherococcus is in the same family as true ginseng, its chemical composition is quite different (Farnsworth et al., 1985; Shibata et al., 1985). We have seen that Eleutherococcus contains a fairly large array of secondary compounds such as lignans, triterpenes, vitamins, etc. and that the majority of secondary compounds isolated from true ginseng are saponins. Indeed, as we have already said, many of the effects of Eleutherococcus are quite unlike those of true ginseng. Even so, extracts of Eleutherococcus do display some effects similar to those of P. ginseng. One is the nominal increase in ability to do physical and mental work. Also, the ability to recover more quickly and fully from serious diseases is also attributed to each of the ginsengs (Brekhman, 1968; Mowrey, 1993). Even so, the vast majority of the pharmacological effects of P. ginseng are quite different from those from E. senticosus. A number of different combinations of true ginseng saponins have been shown to have anti-oxidative, anti-cancer and anti-stress effects in various systems; one saponin (unfortunately, it was not possible for us to match compounds with a ‘Rn’ from Japanese and Chinese papers with a chemical name, even a trivial one.) Rg1 has been reported to alleviate male erectile dysfunction, another has been shown to be active in counteracting adverse effects of morphine and yet another has been reported to act as an inhibitor of tonic pain. A combination of these two saponins has been credited as having an anti-inflammatory effect.

According to modern co-evolutionary theory which describes adaptive changes in plant metabolism upon environmental interactions with herbivorous insects (as well as other animals) and the internal needs of each plant as an organism, Eleutherococcus and true ginseng synthesize compounds such as those listed in Tables 2 and 3 for their own defensive purposes against external hazards and internal degenerative processes (Fu-
tuyma, 1986). Both plants, *E. senticosus* and *P. ginseng*, have many chemical constituents with a very broad variety and range of pharmacological effects. It seems reasonable to suggest that these multiple or pleiotropic effects are due, in part at least, to synergistic interactions between compounds and their binding to various receptors at the cell surface and inside the cells (see also Sanglier, 1993 for a discussion of immunosuppressants from natural sources and how they might work for additional insights.) Eleutherococcus contains various anti-oxidants and these in turn are also immunostimulants and/or immuno-modulants. Long ago, Braun (1969, 1974, 1977) pointed out similarities in etiology and development of cancers between plants and animals. If one accepts the feasibility of the hypothesis that anti-oxidants may be synthesized by *Eleutherococcus* ‘for’ self-defense, then it seems reasonable to speculate that the compounds act similarly when mammalian cells encounter (absorb or otherwise bind) these substances (see e.g. Sies, 1994 and refs. cited therein). Some of the secondary metabolites from *Eleutherococcus* reportedly effective pharmacologically are phenolic compounds associated with gallic acid synthesis (Lewis, 1993). Any of their antagonists would be responsible for anti-oxidative effects on mammalian cells, say in culture. It seems reasonably well established that any of the above-mentioned anti-oxidants synthesized and stored in cells of plants like *Eleutherococcus* can be called upon when an oxidative stress to the plant’s cell(s) occurs. It is less well established whether the levels of anti-oxidant that are effective in the plant are on the same order or higher in animals. Any given animal organism might require a similar, higher or a lower blood plasma concentration for the same anti-oxidative effect. There are no details that we are aware of pertaining to the metabolism of any of the active constituents from *E. senticosus* in an animal organism, certainly not in humans.

Saponins have been reported as secondary metabolites that prevent or detoxify the effect of various diseases in plants (Osborn et al., 1998). We have stated that the chemical constituents of true ginseng are saponins with the same basic structure but with different side chains. These different side chains are apparently responsible for bringing about different pharmacological effects. This is probably because they are the active sites of the saponin molecules. Yet they, like the anti-oxidants from *Eleutherococcus*, display synergistic responses in mammalian cell systems (Yamasaki, 1996).

### 7.3. Adaptogen versus placebo versus panacea?

In order to evaluate the concept of an “adaptogen” by the standards of modern medicine, we compared the term as its main protagonists Lazarev and Brekhman related it to existing pharmacological agents. At the same time, we emphasized that the developers of the adaptogen concept seem not to have determined or specified under which conditions an adaptogen is to be considered being absolutely harmless and innocuous (Brekhman, 1968). In order to so state one obviously would have had to study the effects of a model compound or candidate substance(s), on various systems, its optimal activity, its metabolic degradation and the effects of its products of degradation on an organism.

According to Shapiro and Shapiro (1997), who by contrast carried out extensive studies on placebo effects, a placebo may be defined as follows. “A placebo is any therapy (or that component of any therapy) that is intentionally used for its non-specific, psychological, or psychophysiological therapeutic effect, or that is used for presumed specific therapeutic effect on a patient, symptom, or illness but is without specific activity for the condition being treated...”
“A placebo, when used as a control in experimental studies, is a substance or procedure that is without specific activity for the condition being treated. The placebo effect is the nonspecific psychological or psychophysiological therapeutic effect produced by a placebo” (Shapiro and Shapiro, 1997). Thus it is very clear that the concept of an adaptogen as originally set forth by Lazarev and further developed by Brekhman is very similar to the modern concept of a placebo, which is as ‘non-specific’ in its effects on an organism as the first one. (For yet another perspective of the ‘physiological foundations of the mechanism of action of adaptogens’ readers are referred to (Wagner et al., 1994; Wagner, 1995)).

Some have suggested that an ‘adaptogen’ might be viewed in the context of an immunostimulator that normalizes the immune response of an organism to various stresses (Mowrey, 1993; Rege et al., 1999). This is especially so if one considers an anti-oxidant acting as a protectant against harmful radicals induced by insults from the external environment. Being a protectant as well as having the potential to act synergistically with other anti-oxidants, an adaptogen could indeed serve to treat or even prevent some diseases, or, in the least, participate in the maintenance of levels of critical substances in the body’s fluids etc. (cf. Sies, 1994). This protective activity in turn could help sustain at the clinical level an acceptable level of ‘quality of life’ in the critically ill such as those with AIDS and other immune deficiency syndromes whose immune system is compromised. In those cases there is a characteristic increased production of free radicals and continuous intake of vitamin E and other immunostimulants is necessary (Peterhans, 1994; Jordao et al., 1998). We have, in fact, been able to find one report in which the claim is made that *Eleutherococcus* synthesizes betulinic acid among many other anti-cancerogenic and anti-oxidative compounds (Zhao et al., 1993). Betulinic acid is a reasonably well-studied compound that has been shown to have anti-cancerogenic and anti-viral effects (Yasukawa et al., 1991; Ma et al., 1999). In particular, betulinic acid has been shown to be effective against HIV-1 (human immunodeficiency virus 1) in CEM-4 and MT-4 cell cultures (Vlietinck et al., 1998). On this account, a particular anti-oxidative compound or a combination of compounds could be viewed as offering protection from the effects of harmful radicals from the external environment, as well as exhibiting synergistic effects with other anti-oxidants. In so doing, an ‘adaptogen’ could prevent some ‘new’ disease from developing by helping to maintain a certain level of anti-oxidants in the body fluids and thus help sustain quality of life in chronically ill patients.

Even so, the future of anti-oxidants for the moment does not seem to be particularly bright. For example, human clinical trials do not match encouraging findings from in vitro studies. There are a number of reports that β-carotene allegedly prevents cancer. This seems to be somewhat of an exaggeration. There are a number of different kinds of cancer, of course, and clearly the compound is not a panacea. For example, Greenberg and Sporn (1996) found that β-carotene intake increased the risk of lung cancer in the large clinical group studied; it was not able to normalize early stages of a specific gene responsible for neoplastic development! However, in a different study by Giovannucci et al. (1995) the data showed that lycopene or other compounds in fresh tomato and cooked tomato products had a preventive effect against prostate cancer. Also, Franceschi et al. (1994) showed that raw tomato intake is beneficial in lowering risk against cancers of the digestive tract.

The adaptogenic effect of *eleutherococcus* during and/or after extensive exercise and at high altitude could be explained as the protective anti-oxidative effect of vitamin E and other anti-oxidants contained in plant extracts. That is to say, under such external ‘stresses’ there is increased production of oxygen species as the result of compensation to the lack of oxygen in the outside environment (Packer et al., 1994).

Rätsch (1997) claims that *Eleutherococcus* shows an aphrodisiac effect on animals and has suggested that it should have the same “invigorative” and tonic effect on people. In addition to this, Mowrey (1993) speculated that if *Eleutherococcus* can increase stamina in athletes, it might increase sexual performance as well. The last might be true, especially if one could prove that
phenols from *Eleutherococcus* have effects similar or identical to a 'Viagra effect', a situation in which the substance temporarily inhibits nitric oxide as a result of cyclic GMP signal transduction inhibition (Goldstein et al., 1998). The effect of sesamin, sitosterol and its glucoside, β-sitosterol β-D-glucopyranoside—some compounds with hypocholesteroletic activities—could be explained by their participation in antioxidative reactions on LDL (low density lipoproteins), the accumulation of which causes narrowing and eventual closing of vesicles (Moulton et al., 1999). Therefore, we would argue that sesamin, sitosterol and its glucoside can discharge very specific effects at a molecular level. Another perspective on solving the atherosclerosis problem was reported by Moulton et al. (1999). There, angiogenesis (the formation of vesicles) was achieved in atherosclerotic lesions with inhibitors such as TNP-470 and endostatin. Admittedly, more detailed studies are needed to establish the mechanism of a potential endostatin-like inhibition by sesamin, sitosterol and their glucosides.

We have emphasized throughout that compounds from true ginseng have nominally been shown to have various ‘adaptogenic’ effects. We have seen that true ginseng is widely used as an immune system strengthener in the Eastern Hemisphere. But it is by no means the only plant used for this purpose. Dong Quai (from *Angelica sinensis*, Apiaceae) is said to be second only to true ginseng in its use as a tonic herb, especially amongst women in China. And, favorite ‘tonics’ come and go (c.f. Wagner, 1990, 1995; Wagner et al., 1994 for treatment of several other higher plants with reputed ‘adaptogenic’ activity). Currently there is a fair amount of interest in a ‘Polynesian’ (actually SE Asian and Australasian) plant with ‘remarkable and diverse curative powers’, more specifically touted as an ancient Tahitian medicine, Noni (*Morinda citrifolia* L., Rubiaceae). Dixon et al. (1999) have reported that preparations of Noni (usually the fruit ‘juice’ of *Morinda*) are being used for purposes quite similar to those attributed to true ginseng. Indeed, our detailed study of the literature has verified that true ginseng is reputedly a fortifier of the immune system (Mowrey, 1993), but the evidence in support of this is far from decisive (see e.g. Phillipson and Anderson, 1984; Lewis, 1986; Bahrke and Morgan, 1994 for a critique of some of the studies with true ginseng and the flaws in experimentation).

Clearly the secondary product profile of true ginseng is as complex as that of *Eleutherococcus* and the pharmacology becomes equally complex as a result. Synergistic combination of ginsenosides Rb1 and Rb3, as well as the combination of Rb1 and Rg1 have been reported to have an anti-oxidative effect on conditions brought about on aging neurons associated increased amounts of nitric acid and, as a result, ‘caused’ neurodegenerative diseases such as Alzheimer’s and Parkinson’s (Kim et al., 1998a,d).

It has been suggested that Rg1 alone had an anti-impotence effect supposedly due to its influence on the cell signaling cascade. Unfortunately, this cascade has not been studied closely yet but its reputation has given true ginseng considerable popularity as a powerful aphrodisiac (Fahim et al., 1982; Yoshimura et al., 1998).

PPD (protopanaxadiol) and Rh2 supposedly exhibit an anti-cancerogenic effect due to their antioxidative mechanism of action in which the cell cycle is blocked at the G1 phase and induces apoptosis (Ota et al., 1991; Park et al., 1997; Nakata et al., 1998). Various other ginsenosides (those with various so-called ‘Rns’) have also been reported as being anti-oxidants but it is not clear which and at what concentration those ginsenosides might be responsible for the particular effects (Guogan and Yan, 1997; Kim et al., 1998c).

In sum, the anti-oxidative activities of *Eleutherococcus* and true ginseng constituents of the sort reported above might well qualify to be called ‘adaptogenic’ by some in the sense that they somehow seem to prevent or inhibit undesirable effects of the internal and external environment on/in certain cells and, by extension, on the whole [human] organism. And, it is conceivable but by no means proven that depending on their therapeutic window of activity, some of them might even be so potent that a few doses or even a single dose might decrease cholesterol levels, inhibit
mentally-debilitating processes, or even stop tumors from growing.

One could argue moreover that the activity of a substance may be mono-specific, that is, effective against one particular dysfunction, or poly-specific—that is the effect acts against more than one dysfunction, or beneficial side effects as it were in the western model of a drug. The latter action should not, however, be confused with the effects of panaceas. Panaceas are nominal 'cures' for all or many diseases (from the Greek pan or ‘all’ and akos ‘cure’, that is a panakeia or panacea, thus ‘cure all or universal remedy’). They are, of course, generally discredited in modern western medicine (Root-Bernstein and Root-Bernstein, 1997). It is possible, of course, that the same compound can be active in the treatment of more than one ‘misalignment’. Ibuprofen is but one such example; it acts as an analgesic or painkiller and it is a mild anti-inflammatory agent as well (Reynolds, 1996).

7.4. Problems in the standardization of 'adaptogenic' preparations

A number of field trips to local health food stores and ‘natural pharmacies’ were made in order to find out the range of preparations of *Eleutherococcus* that might be available (see Tables 4 and 5). From this task we learned that most of the currently available *Eleutherococcus* preparations in the metropolitan New York area (and by extension we presume other areas of the USA) are not standardized or are at best, pseudo-standardized. Even a perfunctory examination of the labels showed that labeling as currently practiced leaves much to be desired. Little of the fastidious ‘word-smithing’ reflected in the report of the Panel on Definition and Description, (Complementary Alternative Medicine Research Methodology Conference) April 1995 on definitions and specific materia medica is in evidence (see O’Connor et al., 1997).

The problems of standardization of plant-derived preparations were addressed early in nineteenth century. Resinous podophyllum (the first use of which was as an emetic) seems to be the first plant-derived material to be concentrated. Prof. John King of the Eclectic Medical Institute, Cincinnati (eclecticism was the precursor of modern alternative medicine so far as herbal products are concerned) is credited with the value of the concentration of plant materials. Podophyllum and many other 'concentrated medicines' were marketed after 1847 and became useful among physicians. Concentrated medicines were more palatable and easier for a physician to carry than other botanicals (Rothstein, 1988).

Directions for use of *Eleutherococcus* preparations are generally very brief, and vary from preparation to preparation depending on the manufacturer (cf. Table 4). Few state how much of the preparation should be taken, when it should be taken (e.g. before, during or after a meal), if there are any contraindications that should be taken into account such as incompatibilities with certain foods, prescription or other medications or certain existing health conditions (e.g. hypertension, diabetes) etc.). Indeed, there is increasing evidence that such contraindications should not be taken as merely incidental (Miller, 1998). And, since the popularity in use of herbal preparations is increasing at a rapid rate, it has been argued that health care providers and other
Table 4
Some commercially-available preparations containing E. senticosus or its extractives

<table>
<thead>
<tr>
<th>Name of preparation</th>
<th>Dosage and directions for use</th>
<th>Country of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eku-Kokk-M Dragees</td>
<td>Not indicated</td>
<td>Germany</td>
<td>Internet: <a href="http://www.tee.org/BHSD/bhsd149.html">http://www.tee.org/BHSD/bhsd149.html</a></td>
</tr>
<tr>
<td>‘Eleutherococcus cararina’ Tropfen [drops]</td>
<td>Not indicated</td>
<td>Germany</td>
<td>Internet: <a href="http://www.tee.org/cgi-bin/BHSD-serverseqenum">http://www.tee.org/cgi-bin/BHSD-serverseqenum</a> = 49 &amp; weiter = x</td>
</tr>
<tr>
<td>‘Konstitutin Forte’ Kapseln [capsules]</td>
<td>Not indicated. (A) Take 2 ml of extract 30 min before meals. (B) Take 29–30 drops as indicated above</td>
<td>Germany</td>
<td>Internet site as above (A) Sokolov et al., 1990. (B) Mashkovsky, 1988</td>
</tr>
<tr>
<td>Standardized®, Full Potency™ Siberian Ginseng Root Extract Vegicaps®</td>
<td>Not indicated</td>
<td>New Jersey, USA</td>
<td>Solgar Vitamins and Herb Company, Catalog</td>
</tr>
<tr>
<td>Nature’s Way. ‘Wild Siberian Ginseng Root’</td>
<td>Two or three 410 mg capsules three times a day with water at mealtimes.</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Enzymatic Therapies®, Siberian Ginseng Extract. Natural herbal extracts standardized to contain &gt;1% eleutheroside E</td>
<td>Take one 100 mg capsule three times a day as an additive to everyday diet.</td>
<td>Wisconsin, USA</td>
<td>Developed in accordance and with recommendations and safety standards by German Commission E. User referred to: Hotline telephone number +1-800-7832286 Certified Siberian ginseng extract, standardized for a minimum of $B = 400$ mcg, $D = 300$ mcg per capsule in a synergistic base of wild countryside Siberian ginseng. Product label and Internet: <a href="http://www.futurebiotics.com">http://www.futurebiotics.com</a></td>
</tr>
<tr>
<td>Natures Herbs®, Power Herbs. Siberian Ginseng</td>
<td>Take two 400 mg capsules 2-3 times a day with water. (100 mg of b and d eleutherosides per capsule)</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Futurebiotics. Premium Blend Standardized Korean and Siberian Ginsengs</td>
<td>As a dietary supplement for adults. Take one capsule a daily or as directed by a healthcare practitioner. (20% ginsenosides and 0.5% eleutherosides; 500mg/capsule = 300 mg ginsenosides+200 mg eleutherosides)</td>
<td>New York (Long Island), USA</td>
<td></td>
</tr>
<tr>
<td>Natrol, Four Ginsengs. Guaranteed Potency Extract. A dietary supplement capsules. One capsule contains: Panax ginseng extract (32% ginsenosides) 100 mg; Panax quinquefolium (5% ginsenosides) 100 mg; Eleutherococcus senticosus extract (8% eleutherosides) 100 mg; Devil’s Club = Oplopanax horridum (Incorrectly given as Oplopanax) 50 mg)</td>
<td>Take One or two capsules twice a day.</td>
<td>Utah, USA</td>
<td>Internet: <a href="http://www.natrol.com">http://www.natrol.com</a>. and product label</td>
</tr>
<tr>
<td>Nature’s Herbs®, A Twinlab Co.® Siberian Ginseng Root (404 mg/capsule)</td>
<td>As an additive to the daily diet: 2 to 3 capsules three times a day with a large glass of water.</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Name of preparation</td>
<td>Dosage and directions for use</td>
<td>Country of origin</td>
<td>Reference</td>
</tr>
<tr>
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<td>-----------------------------</td>
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</tr>
<tr>
<td>Fast activities®, Nature’s Way. Wild Siberian Ginseng Root alcohol extract. Siberian ginseng root extracted in pure grain alcohol (35-45%) and spring water. Contains 14% deuterolides B and E; 1.4 mg deuterolides: 1 ml liquid extract.</td>
<td>Shake well before use. Take 2 ml (for adults and ½ adult dosage for children under 12), three times a day for 8 weeks followed by a 2 week break</td>
<td>Utah, U.S.A.</td>
<td>Product label</td>
</tr>
<tr>
<td>Zand®, Active Herbal®, Siberian Ginseng Formula for an Active Life Style</td>
<td>Take one teaspoon (5 ml) three times a day. May be taken as often as six times a day during periods of intense work and prior to athletic competition. Shake well before using.</td>
<td>California, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Galia Herbs Inc.® Fresh Plant Extracts, Wild ginseng supreme, wild American ginseng, wild Russian ginseng in grain alcohol 30-35%. (1 fl. oz.) Average fresh herb strength 1:15.</td>
<td>Suggested use: thirty to forty drops in a small amount of warm water 3-4 times a day between meals.</td>
<td>Massachusetts, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Alvita®, Caffeine Free Siberian Ginseng Tea bags 1.69 oz (48 g)</td>
<td>Place one tea bag in a cup, add no more than 6 oz. of boiling water, steep for three minutes, remove bag, press bag before removing to enhance the flavor. Add honey to sweeten.</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Fast Activities® Nature’s Way®, Wild Siberian ginseng root glycerine extract 59 ml (2 fl. oz.), Total Spectrum™ Extract. Siberian ginseng root extracted in (kosher) glycerine and spring water. Potency: 500 mg dry root: 1 ml liquid extract. Double maceration.</td>
<td>Shake well, adults 2 ml three times a day for eight weeks followed by a two weeks break; children under 12 take ½ adult dosage. May be added to food or beverage. Refrigerate after opening.</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
</tbody>
</table>

* The preparations listed here were of course available on the market when the table was prepared. Experience has shown, however, that manufacturers and suppliers and indeed Websites for the whole range of herbal preparations change rather frequently. Thus, the listings do not imply availability. Moreover, it should be noted that the FDA (Food and Drug Administration of the USA) requires a disclaimer regarding these products to the effect that ‘The statements have not been evaluated by the FDA. These products are not intended to diagnose, treat, cure or prevent any disease’. No endorsement or otherwise of any brand is implied or intended.

* It is not clear how the preparation is standardized. This preparation, as well as others sold by this company, is said to represent a standardized exact and powdered raw material of plant roots. Solgar makes the claim that the plant is prepared so ‘the result is a potent, premium-quality traditional herbal extract with all of the synergistic and beneficial qualities found in nature’. Finally, it is worth mentioning that E. senticosus used to be sold in the USSR in the form of a popular non-alcoholic drink ‘Bodrost’; and also as an ingredient in one brand of vodka.
Table 5
Some commercially available preparations containing Panax gingseng or its extractives

<table>
<thead>
<tr>
<th>Name of preparation</th>
<th>Dosage and directions for use</th>
<th>Country of origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tinctur Ginsengia^</td>
<td>15–20 drops 3 times a day before meals</td>
<td>CIS</td>
<td>Sokolov et al. (1990)</td>
</tr>
<tr>
<td>Tablets or powder of Panax ginseng</td>
<td>0.15–0.3 g three times a day before meals</td>
<td>Same as above</td>
<td>Same as above.</td>
</tr>
<tr>
<td>Standardized. Full Potency Korean™</td>
<td>No dosage or directions for use indicated</td>
<td>New Jersey, USA</td>
<td>Solgar Vitamins and Herb Company, Catalog</td>
</tr>
<tr>
<td>Ginseng Root Extract Vegicaps®</td>
<td>Take three capsules a day with a large glass of water</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Nature’s Herbs. Korean Ginseng Root (556</td>
<td>As an additive to diet. Adults: one capsule twice a day with a</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>mg/capsule) contains countryside Korean</td>
<td>large glass of water</td>
<td></td>
<td></td>
</tr>
<tr>
<td>white ginseng root in a preservative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>capsule</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nature’s Herbs® Power-Herbs® Korean</td>
<td>Take one-two capsules a day as an additive to the everyday diet.</td>
<td>Wisconsin, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>ginseng power-herb® (7% ginsenosides, 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg extract/capsule; 530 mg capsule)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzymatic Therapy® Panax ginseng</td>
<td>As beverage: take two capsules each time after meals or as directed</td>
<td>USA</td>
<td>Product label</td>
</tr>
<tr>
<td>root extract, 100 mg standardized to</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>contain 7% saponins calculated as</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ginsenoside Rg1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic Natural. Pacific Brand. The</td>
<td>Suggested use: shake well, dilute 0.5–1.5 ml in a cup of liquid, 2–3</td>
<td>California, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>Queen of Herbs in Orient Korean Ginseng</td>
<td>times a day or as needed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>for tea (648 mg/capsule, 10 grains)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zand®. Premium Standard™ Chinese ginseng</td>
<td>Take one tea bag in a cup and add no more than 6 oz of boiling</td>
<td>Utah, USA</td>
<td>Product label</td>
</tr>
<tr>
<td>(25 g). Contains Panax ginseng, distilled</td>
<td>water, steep for 3 min, remove bag, press bag before removing to</td>
<td></td>
<td></td>
</tr>
<tr>
<td>water, grain alcohol (ethyl alcohol USP</td>
<td>enhance the flavour. Add honey to sweeten</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40–45%). Extract contains 10.3 mg of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ginsenosides/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alvita®. Caffeine free Panax ginseng tea</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bags 42 g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^ It is worth mentioning that many preparations available in the USA seem not to have detailed dosage indications unlike their counterparts from Britain, CIS, Germany, etc.

medical personnel should feel obligated, even if they are not legally mandated, to advise the public on proper use of such medicines (Zink and Chaffin, 1998).

The 103rd Congress, 2d Session (US Congress, 1994) provided guidelines for dietary supplements. However, our Table 4 clearly shows that at the time of completion of this manuscript, labels of preparations that are available to a customer are indeed often very attractive (see Fig. 4 a, b as two randomly selected examples. But labelling remains for the most part general and detailed information on use may be lacking. In many cases, the chemical nature and quantity of purportedly ‘active’ ingredient(s) are missing. In short, standardisation, if any, appears minimal. Potential users would be hard pressed, we would argue, to make a rational selection. True ginseng preparations are not much better (cf. Table 5).

It is not uncommon among users of various medications not only to take a drug whenever there is a ‘problem’ but equally often to take more
of it than otherwise might be indicated or prescribed, since there is the widespread perception that if something ‘works’ at a low dose level, then it should work better still if a higher dose is utilized. Attention has been drawn to this tendency in a slightly different context by Sahelian in his small book “Kava. The Miracle Antianxiety Herb” (Sahelian, 1998). Although a physician, Sahelian encourages users to increase the dosage of their kava intake if a given level seems not to ‘work’. By recommending what one might refer to as a personally-determined ‘titration’ to achieve a desired effect, Sahelian admits that the preparation might not work on a certain individual (cf. Table 6). Of course, this could be due to a number different reasons, such as dosage of a particular drug should be determined according to strength of the preparation, one’s body weight, sex, age, peculiarities like attendant chronic diseases which can alter the drug’s metabolism, concomitant utilization of other medications etc. Even the ability of the patient’s digestive system to degrade a product to liberate bioactive low molecular weight compounds from repeating polymers has been offered as a reason for poor activity in some users (Niwa et al., 1991). In other words, the value of individualized doses has long been recognized in pharmacy and pharmacology. Yet another factor that candidly admits the deplorable state of standardization of many herbal supplements is that one has to even take into account whether the stated ingredients really are reflected by the contents of the marketed product. For instance, the amount of ginsenosides disclosed upon analysis of various ginsengs and ginseng preparations have been shown to vary tremendously (cf. e.g. Liberti and DerMarderosian, 1978; Ziglar, 1979; Lui and Staba, 1980).

8. Conclusions

*Eleutherococcus senticosus* gained considerable advocacy and use among a fairly broad sweep of the Soviet population after its introduction to the general Soviet medical community by the researchers in Siberia. Significantly, it never gained very wide popularity outside the Soviet bloc or sphere of influence. In the West it has usually been characterized (incorrectly to be sure) as a weaker, much less effective substitute for true ginseng (Fulder, 1980b; Tyler, 1994). Some reasons for the apparent lack of wider appreciation of *Eleutherococcus* may be mentioned. First of all, virtually all of the early work on *Eleutherococcus* was published in foreign journals and scholarly monographs and books, mostly in Russian, some in German and Chinese and few in Ukrainian. Lack of ready access to this primary literature or ability of many medical scientists or chemists to read it certainly has served as a deterrent to those in other countries who might have advocated its use or undertaken independent studies on the plant. The general unavailability, more accurately the perceived lack of a reliable supply, of *Eleutherococcus* because of its growth in areas like the
eastern Soviet Union, especially Sakhalin, and the People’s Republic of China, must have also played a role in discouraging potential, more mainstream scientists, especially western investigators from getting involved. We have seen that most of the studies of the effects of *Eleutherococcus* preparations on human subjects, along with experiments on animals, were made by Brekhman et al. in the 1960s to the 80s in the former USSR. And, Russian scientists in the 1950s to the 60s also carried out much of the isolation of active compounds from both *Eleutherococcus* and true ginseng. Later, additional chemical work on the isolation of various constituents and studies on their chemistry were done by German (cf. e.g. Wagner et al., 1982; von Wagner et al., 1984), Chinese (cf. e.g. Fang et al., 1985) and Japanese scientists (cf. e.g. Hikino et al., 1986).

As to the relative lack of enthusiasm in the development of *Eleutherococcus* as a western-style pharmaceutical or drug preparation, one can say the following. Of course, different approaches exist around the world as to the process of approval of certain substances from plants intended for medicinal use (Lawson and Bauer, 1998). This fact has also served to exacerbate any existing scientific cultural information gap between development and use of pharmaceuticals in different countries, and this must be construed as another factor in the failure of *Eleutherococcus* to be studied by more workers in the West. Indeed, the substantial financial support required for carrying out laboratory studies, and the fairly long lead-time required for even the chemical work with plants is a real issue. At the clinical level, most experiments utilizing plants suggest that it requires a fairly long time (weeks, months) for the pharmacological effects to become manifest. This is understandable if one takes into account that the levels of secondary constituents are usually not very high, at least in those plants that do not show extreme toxicity. (Parenthetically, some Russian scientists did seek to ascertain when optimal levels of the active constituents in crude *Eleutherococcus* were produced by testing material throughout the year and showed that the highest concentrations of eleutherosides were to be found in the spring- and fall-harvested material. However, of greatest concern to them were only the lignans, specifically eleutherosides B, D and E (syringin, (+) and (-) syringoresinol, respectively, Lapchik and Ovodov, 1969). It would be very useful to quantify the production of these and other potentially useful secondary metabolites mentioned in this paper as has begun to been done in the case of *Sesamum indicum* L. (sesame) (Ogasawara et al., 1998). Likewise the intermediary metabolism of the substances in the producer plants therefore needs to be studied. Such knowledge could be very useful in understanding how new drugs could be developed that are more finely tuned to the needs of a given patient (cf. Table 6). Simultaneously, it must be recognized that the proportions of the diverse constituents present in botanical medicines might have a major role in determining efficacy and even safety. It could end up that if proportionality is altered significantly, over-consumption of any given component(s) could lead to lack of activity, altered activity or even toxicity. Incomplete information suggests that caution must be exercised. Indeed, preparations hitherto thought to be safe may turn out to be unsafe if concentrated preparations are used, or even genetically engineered specimens are generated with the nominal desire to increase production of the ‘active’ metabolite(s).

Biosynthesis of ginsenosides (true ginseng saponins) by cultured cells of *P. ginseng* has been reported. However, the decomposition of ginsenosides apparently gets activated periodically and cleavage occurs leaving carbohydrate residues. For this reason, the amount of ginsenosides may vary in different parts of the plant as well as in different organelles and compartments of different cells of the same plant (Konstantinova et al., 1995).

All the above emphasizes that in the modern, highly competitive scientific world of pharmaceutical research, it seems to be unappealing to invest time and money in activities where fairly quick results are not obtainable. (See Grabley and Thiericke, 1999 for an in-depth treatment of various new strategies and approaches aimed at facilitating natural drug discovery.) Or, as some have suggested, the potential for patent rights is not patently assured (cf. e.g. Patel, 1998; Frieden, 1999 for a ‘pro’ view and Barrett, 1998 for a ‘con’ position on this feature of natural products patents).
Despite all the above, and with the full benefit of retrospection, the ‘adaptogen concept’ and the concept of ‘adaptogenic’ substances as originally espoused by the Soviets seems not to be the best (or even an appropriate) medico-scientific context for assessing, quantifying and promulgating the virtues of the pharmacological activity or medicinal potential of *Eleutherococcus*, or of any other plant for that matter. Also, it is important to recognize that the reporting of results from studies on eleutherococcus chemistry were often as confusing and imperfect as the erection of the ‘adaptogen’ concept. Nevertheless, as imperfect and incomplete as the adaptogen concept and the (bio)chemistry underlying it have been, and still are, it has been possible for us to pull out of the literature a number of very interesting and seemingly valid reports where more than one active compound present in *Eleutherococcus* has been tested alone or in combinations that can yield important, indeed, novel, synergistic responses, some of which have been carefully documented in the peer-reviewed literature.

Since specific combinations of synergistically acting substances produce a particular pharmacological effect, it follows that those molecules having the synergistic effects must participate in several reactions. We are quick to re-emphasize that there is a large complex of chemical components in *Eleutherococcus* and with it, comes the inherently high potential for bringing about inter-actions, potentiations and/or combinatorial responses. These complex responses have been very inadequately studied and will probably not be easy to sort out (Nies and Spielberg, 1996). Admittedly the literature that reports such a wide array of seemingly unrelated pharmacological responses often gives the impression that the studies have been superficial and incomplete and often have the ring of testimonials attesting to panacea-like effects. Last, but not least, it is clear that the inability to provide a distinctive, scientifically unassailable definition of an adaptogen seems to have caused many practitioners of ‘conventional’ or ‘allopathic’ western medicine to dismiss the adaptogenic concept as symbolic of ‘fringe medical practice’ so often characterized by use of disreputable ‘nostrums’. (Incidentally, the same may be said of homeopathic medicines Kollerstrom, 1982; Morowitz, 1982).

We hope that as a result of our analysis of the literature that one can now better appreciate the kinds and range of physiological and pharmacological effects of *Eleutherococcus* (and true ginseng, *P. ginseng*). We believe that along with the phytochemical work, although admittedly still relatively incomplete, the results reported to date allow us to conclude that adaptogens, whether so designated or not (and we definitely suggest they not be so designated) have the potential to work against various forms of ‘illness’.

We have tried to identify and address the problems associated with adaptogens and have attempted to provide a more precise context for understanding them better. We believe that the phytochemical profile, i.e. the presence of various chemical constituents in *E. senticosus* and their demonstrated pharmacological action in a wide range of tests, suggests that one can advocate without reservation further research into this plant. Only by this means will their potential maximal value be substantiated (or discredited) and their worth as models for development of still more effective medicines be taken advantage of. It is certainly more than a little suggestive that many, if not most, of the substances extant as secondary metabolites in plants such as *Eleutherococcus* and true ginseng bear strong resemblance to products that are productions of human metabolism. For example, attention may be drawn to the similarity of substances like saponins and cholesterol, lignans and nucleosides. Might these phytochemicals not be legitimately viewed as molecular analogs of various compounds regularly produced in the course of human anabolism and catabolism? One could speculate still further by pointing out that compounds like AZT and acyclovir are nucleoside analogs, as is syringin. Incorporation into DNA could prevent DNA damage from radiation; the hypocholesterolemic effect of sesamin could be due to a direct effect on de novo cholesterol synthesis; the anti-cancer effect of sesamin, as well as coniferyl aldehyde and caffeic acid ester could likewise be due to incorporation into DNA; the detoxifying effect of sesamin on ethanol could well be due to its effect
on its solubility. The anti-inflammatory effect of sitosterol could be due to an effect much like that of aspirin on prostaglandin. The hypocholesteremic effect of sitosterol might well be due to a mimicking of cholesterol’s structure and further inhibition of cholesterol metabolism in the human. And, the anti-hyperglycemic action of sitosterol and its glucoside could be explained by its competitive inhibition of enzyme in glucose breakdown. There is no end to the possibilities. We believe what we have presented on *Eleutherococcus* (and true ginseng as well) is robust and relatively complete. But we readily admit that this analysis is only a point of departure for more focused research.

Last, but not least, in addition to recommending that the terms adaptogen or adaptogenic activity be dropped and replaced with more limited, precise descriptors that can be defended scientifically, we believe that it would be helpful if the designation ‘Siberian ginseng’ be dropped in favor of the more accurate (and adverse-connotation or baggage-free) designation of *Eleutherococcus*. The two plants *P. ginseng* and *E. senticosus* have far less in common than has hitherto been implied or supposed both in terms of the chemistry of their secondary products and their pharmacological activities.

Acknowledgements

We appreciate the help of Mary Lou Quinn, Managing Director, NAPRALERT (NAtural PROducts ALERT), Program for Collaborative Research in Pharmaceutical Sciences, (a World Health Organization Collaborating Centre for Traditional Medicine), College of Pharmacy, University of Illinois, Chicago for making available the titles on *Eleutherococcus* that are in that data base. We also acknowledge the help rendered by the staff of the Health Sciences Library at Stony Brook Medical Center. The authors are especially grateful to Donna Sammis of the Interlibrary Loan Department of the Stony Brook University Library for her expert help in obtaining for us many articles from Russian (and other) language journals and books not available on this campus.

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Antimicrobial, antioxidant, antitumour-promoting and cytotoxic activities of different plant part extracts of *Garcinia atroviridis* Griff. ex T. Anders.

M.M. Mackeen a, A.M. Ali a,*, N.H. Lajis b, K. Kawazu c, Z. Hassan d, M. Amran b, M. Habsah b, L.Y. Mooi a, S.M. Mohamed a

a Department of Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
b Department of Chemistry, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
c Laboratory of Bioresources Chemistry, Faculty of Agriculture, Okayama 700-8530, Japan
d Department of Food Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Received 15 July 1999; received in revised form 8 November 1999; accepted 30 March 2000

Abstract

Crude extracts (methanol) of various parts, viz. the leaves, fruits, roots, stem and trunk bark, of *Garcinia atroviridis* were screened for antimicrobial, cytotoxic, brine shrimp toxic, antitumour-promoting and antioxidant activities. The crude extracts exhibited predominantly antibacterial activity with the root extract showing the strongest inhibition against the test bacteria at a minimum inhibitory dose (MID) of 15.6 μg/disc. Although all the extracts failed to inhibit the growth of most of the test fungi, significant antifungal activity against *Cladosporium herbarum* was exhibited by most notably the fruit (MID: 100 μg), and the leaf (MID: 400 μg) extracts. None of the extracts were significantly cytotoxic, and lethal towards brine shrimps. The root, leaf, trunk and stem bark extracts (except for the fruits) showed strong antioxidant activity exceeding that of the standard antioxidant, α-tocopherol. Antitumour-promoting activity (> 95% inhibition) was shown by the fruit, leaf, stem and trunk bark extracts. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Garcinia atroviridis*; Antimicrobial; Antioxidant; Antitumour-promoting

1. Introduction

*Garcinia atroviridis* Griff. ex T. Anders (Guttiferae), locally named ‘asam gelugor’ in Malay, is a medium-sized fruit tree endemic to Peninsular Malaysia. This species grows wild throughout Peninsular Malaysia but is also widely cultivated especially in the northern states owing to its economic and medicinal value. Sun-dried slices of the fruits, locally known as ‘asam keping’, are commercially available and are popularly used as a seasoning in curries, sour relish and also for dressing fish (Burkill, 1966; Corner, 1988). Occasion-
ally, the young leaves are used for culinary purposes and as a traditional vegetable (‘ulam’; Mackeen et al., 1997a). In the East Coast states of Peninsular Malaysia, fresh fish are steamed with the leaves of *G. atroviridis* to delay spoilage (personal communication). In folkloric medicine, *G. atroviridis* is used as post-partum medication and to treat earache, throat irritation, cough, dandruff, and stomach pains associated with pregnancy (Burkill, 1966; Fui, 1992; Grosvenor et al., 1995a).

Extracts of the *Garcinia* genus, particularly *Garcinia mangostana* and *Garcinia kola*, have been extensively reported to exhibit diverse biological activities such as anti-HIV, antimicrobial, antihepatotoxic, antioxidant, anti-inflammatory, and antiulcerogenic activities, but only a few of these studies involved *G. atroviridis*. From the investigations of the antimicrobial, antinematodal, antitumour-promoter, and antiviral activities of *G. atroviridis*, only antibacterial activity was found to be significant (Grosvenor et al., 1995b; Mackeen, 1995; Murakami et al., 1995; Mackeen et al., 1997a,b). Although the occurrence of xanthones, benzophenones and biflavonoids is common in the *Garcinia* genus, to date, the isolation of only atroviridin, garcinia acid (identical to synthetic (−)-hydroxycitric acid) and its γ-lactone has been reported (Lewis and Neelakantan, 1965; Waterman and Hussain, 1983; Kosin et al., 1998). Hydroxycitric acid, which has been primarily obtained from the *Garcinia* genus, is an effective inhibitor of lipogenesis with commercial and clinical applications (McCarty, 1995; Moffett et al., 1996).

In view of the ethnobotanical reports of *G. atroviridis* being used in folkloric medicine particularly as a potent anti-infective agent and biopreservative coupled with the paucity of studies on the biological activities, the present study was undertaken to further characterise the biological activities of *G. atroviridis*.

### 2. Materials and methods

#### 2.1. Plant material

The fruits, roots, leaves, trunk and stem barks of *G. atroviridis* Griff. ex T. Anders. (Guttiferae) were collected in Serdang, Selangor. The herbarium voucher specimen (MM-1) was identified by A. Sivarimuthu, Department of Biology, Universiti Putra Malaysia and deposited at the herbarium of the same department.

#### 2.2. Extraction of various parts of *G. atroviridis*

The fruits, leaves, roots, trunk and stem barks (5 g each) of *G. atroviridis* were separately soaked in methanol (25 ml) for 2–3 days. The yields of the extracts were recorded as weight (g) of crude methanol extract per 100 g of fresh plant material as indicated in Table 1(B). The methanol extracts of the different plant parts were then evaporated under reduced pressure and dissolved in MeOH-DMSO (9:1) to prepare stock solutions of 200 mg/ml. These stock solutions were used in all the bioassays unless mentioned otherwise.

#### 2.3. Microorganisms

The bacterial and fungal stock cultures were maintained on nutrient agar (Oxoid, UK) and potato dextrose agar (Oxoid, UK) slants, respectively, which were stored at 4°C except for *Cladosporium herbarum* which was maintained at 27°C and subcultured every month. Seven microbial strains, i.e. *Bacillus subtilis* B28 (mutant; Gram-positive), *B. subtilis* B29 (wild-type; Gram-positive), methicillin-resistant *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative), *Pseudomonas aeruginosa* UI 60690, *Candida albicans* (yeast) and *Aspergillus ochraceus* ATCC 398 (fungus), and three phytopathogenic fungi, i.e. *C. herbarum*, *Fusarium moniliforme* and *Alternaria* sp., were used for the antimicrobial assay. For the purpose of antimicrobial evaluation, the microorganisms were cultured in nutrient broth for bacteria and potato dextrose broth for fungi at 30°C overnight (excluding *C. herbarum*, *F. moniliforme* and *Alternaria* sp.). The concentrations of the cultures were adjusted turbidimetrically at a wavelength of 600 nm to $10^5 – 10^6$ colony forming units (CFU) per ml.
2.4. Culture of cells

The CEM-SS (human T-lymphoblastic leukaemia) cell line was obtained from the National Cancer Institute, USA and the Raji (human B-lymphoblastoid) cell line was provided by Professor K. Koshimizu, Kinki University, Japan. Cells were cultured in RPMI-1640 (Sigma, USA) medium with 10% v/v foetal calf serum (Sera Lab, UK), 100 IU/ml penicillin (Sigma, USA) and 100 μg/ml streptomycin (Sigma, USA) as a complete growth medium (CGM). Cells were maintained in 25 cm³ flask with 10 ml of CGM at 37°C with 5% CO₂. Every 3 days the cells were subcultured by splitting the culture with fresh CGM at a ratio of 2:8.

2.5. Cytotoxicity assay in microtiter plates

Cytotoxicity was determined using the MTT assay reported by Mosmann (1983). Varying concentrations of the plant extracts were prepared from the stock solutions by serial dilution in RPMI-1640 to give a volume of 100 μl in each well of a microtiter plate (96-well) as described before (Ali et al., 1996). Each well was filled with 100 μl of CEM-SS cell suspension in CGM at 1–2 × 10⁵ cells/ml. Controls that contained only CEM-SS cells were included for each sample. The assay for each concentration of extract was performed in triplicate and the culture plates were kept at 37°C with 5% (v/v) CO₂ for 3 days. After 72 h of incubation, 100 μl of medium was removed from each well. Subsequently, 20 μl of 0.5% w/v MTT (Sigma, USA), dissolved in phosphate buffer saline, was added to each well and allowed to incubate for a further 4 h. After 4 h of incubation, 100 μl of 1 N hydrochloric acid/isopropanol (1:24) was added to each well of the microtiter plate.

Table 1

<table>
<thead>
<tr>
<th>Table 1: Antimicrobial activity of <em>G. atroviridis</em> extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A) Antibacterial activity</strong></td>
</tr>
<tr>
<td>Crude extract</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Fruits</td>
</tr>
<tr>
<td>Leaves</td>
</tr>
<tr>
<td>Stem bark</td>
</tr>
<tr>
<td>Trunk bark</td>
</tr>
<tr>
<td>Roots</td>
</tr>
</tbody>
</table>

<p>| <strong>(B) Antifungal activity</strong>                                   |</p>
<table>
<thead>
<tr>
<th>Crude extract</th>
<th>Yield (w/w)</th>
<th>MID (μg/disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruits</td>
<td>5.8</td>
<td>100</td>
</tr>
<tr>
<td>Leaves</td>
<td>4.5</td>
<td>400</td>
</tr>
<tr>
<td>Stem bark</td>
<td>6.4</td>
<td>&gt;800</td>
</tr>
<tr>
<td>Trunk bark</td>
<td>4.2</td>
<td>&gt;800</td>
</tr>
<tr>
<td>Roots</td>
<td>8.4</td>
<td>&gt;800</td>
</tr>
</tbody>
</table>

* B28 = *B. subtilis* (mutant).
* B29 = *B. subtilis* (wild-type).
* MRSA, methicillin-resistant *S. aureus*.
* Extracts of the fruits, leaves, stem bark, trunk bark, and roots were inactive at the highest concentration tested against the fungi *A. ochraceus*, *C. albicans*, *F. moniliforme* and *Alternaria* sp. (MID: >1000 μg/disc).
* Weight (g) of crude methanol extract per 100 g of fresh plant material.
and vigorously mixed to dissolve the formazan crystals. Absorbance values at 550 nm was measured with a microplate reader (Bio Tek EL 340, USA) after background subtraction at 630 nm. Cytotoxicity was expressed as CD$_{50}$, i.e. the concentration to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells).

2.6. Brine shrimp lethality assay

Brine shrimp (Artemia salina Leach) eggs (Gold Eagle, USA) were placed in a hatching tank containing sea water for 48 h (Meyer et al., 1982). Each extract (20 mg) was dissolved in 2 ml methylene chloride:methanol (1:1) to prepare a stock solution of 10 mg/ml. From the stock solution, 500, 50 and 5 ml were transferred to different vials and allowed to evaporate. After evaporation, 5 ml of brine was added to each vial to prepare concentrations corresponding to 1000, 100 and 10 µg/ml. Each concentration was prepared in triplicate. Ten shrimp nauplii were added to each vial (30 shrimps per concentration). The number on survivors out of 30 shrimps per concentration was recorded and the LC$_{50}$ values ($P < 0.05$) were calculated using the Finney computer programme.

2.7. Antimicrobial assays

2.7.1. Disc diffusion method

An even spread of microorganisms was prepared by mixing 200 µl of inoculum (adjusted to $10^5$–$10^6$ CFU/ml) with 20 ml of agar at 50°C and allowed to set (NA for bacteria and PDA for fungi) in a petri dish ($φ$ 90 cm) (modified from Bauer et al., 1966). One milligram of extract was loaded onto each Whatman No. 1 filter paper discs ($φ$ 6 mm) and placed on the previously inoculated agar. The plates were inverted and incubated for 24 h at 30°C. Antimicrobial activity was indicated by the presence of clear inhibition zones around the discs. For quantitative determination of antimicrobial activity, the minimum inhibitory dose (MID), i.e. the minimum dose per disc to inhibit growth of the test microorganism, was recorded. To determine the MID, serially diluted (two-fold) concentrations of extracts that showed positive activity during the preliminary screening were loaded onto filter paper discs and assayed as described above.

2.7.2. Spore germination method

Ten milliliters of sterile 0.85% (w/v) saline solution was poured onto the agar plate ($φ$ 90 cm) of a confluent culture of F. moniliforme and Alternaria sp. The surface of the culture was gently rubbed with an inoculating loop to harvest the spores. The resulting spore suspension was transferred to a sterile centrifuge tube. The spore suspension was centrifuged at 2000 rpm for 20 min. The supernatant (0.5 ml) was well-mixed with 20 ml of PDA at 50°C and allowed to set in a petri dish. Filter paper discs containing the extracts were placed on the surface of the previously inoculated agar. The petri dishes were inverted and incubated at 27°C for 48 h. Antifungal activity was indicated by the presence of clear inhibition zones around the discs. MID values were determined as described above.

2.7.3. Bioautography using C. herbarum on TLC

Spray medium (0.1% agar medium, 20 ml) was added to well-sporulating agar slant cultures of C. herbarum (Homans, and Fuchs, 1970). The surface of the culture was rubbed with a paint brush to release the spores. The collected spore suspension was passed through gauze before being transferred into a sprayer. Serial dilutions of the extracts spotted on a TLC sheet (Merck 5748, Germany) for MID determination were sprayed adequately with the spore suspension. Sprayed TLC sheets were incubated in a wet chamber at 27°C for 24 h in the dark. Antifungal activity appeared as clear inhibition zones against the dark background of C. herbarum spores.

2.8. In vitro antitumour-promoting assay

The inhibition of Epstein Barr virus (EBV) activation was used to evaluate in vitro antitumour-promoting activity (Ohigashi et al., 1986). Raji cells were incubated for 48 h at 37°C under 5% CO$_2$ in 1 ml ($\sim 5 \times 10^5$ cells) of RPMI 1640 medium containing 3 mM sodium n-butyrate (Nacarai Tesque, Japan), 50 nM phorbol 12-
myristate 13-acetate (PMA; Sigma, USA) and the test extracts (200 μg/ml) per well of a 24-well plate. Conventional indirect immunofluorescence staining of early antigen (EA) with high-titre EA-positive serum (EA titre 1:1280) obtained from NPC patients (1:20 serum dilution) followed by a 1:30 dilution of fluorescein isothiocyanate (FITC)-labeled IgG (Sigma, USA), was used to measure EBV activation. The ratio of EA-induced cells to the control experiments only with sodium n-butyrate and PMA was around 30%. Extracts showing antitumour-promoting activity were serially diluted (two-fold) to determine the IC_{50}, i.e. the concentration to inhibit tumour promotion by 50%.

2.9. Antioxidant assays

2.9.1. Ferric thiocyanate (FTC) method

A screw-cap vial (φ 38 × 75 mm) containing a mixture of 4 mg (4 ml) of a sample (final concentration, 0.02%) in 99.5% ethanol, 4.1 ml of 2.5% linoleic acid (TCL, Japan) in 99.5% ethanol, 8.0 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in an oven at 40°C in the dark (Kikuzaki and Nakatani, 1993). To 0.1 ml of this mixture, 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% ammonium thiocyanate (Wako, Japan) solutions were added to 2.0 ml from the mixture (containing sample) prepared in the FTC method (Ottolenghi, 1959). This mixture was kept in a water bath (100°C) for 10 min and, after cooling to room temperature, was centrifuged at 3000 rpm for 20 min. Antioxidant activity was based on the absorbance of the supernatant at 532 nm on the final day of the FTC assay.

3. Results and discussion

The antimicrobial activity and yield of G. atroviridis extracts is presented in Table 1. The root extract showed exceptionally strong antibacterial activity against all the bacteria at the MID of 15.6 μg/disc which was at least eight-fold stronger than the next most active extract. The fruit, leaf and root extracts exhibited broad-spectrum antibacterial, i.e. to both Gram-positive (B. subtilis B28 & B29, MRSA, S. aureus) and Gram-negative bacteria (E. coli and P. aeruginosa). The stem and trunk bark extracts inhibited all the Gram-positive bacteria but inhibited only P. aeruginosa among the Gram-negative bacteria. The stem and trunk bark extracts were most active against the B. subtilis B29 and P. aeruginosa, respectively, at 125 μg/disc. Comparison of the susceptibility of all the bacteria to the various extracts indicated that E. coli was the most resistant because its growth was inhibited only by the extracts from the fruits and particularly the roots (32-fold stronger than the fruits).

The insignificant difference in the inhibitory action of all the extracts against both the mutant (DNA-repair deficient) and wild strains of B. subtilis ruled out the involvement of DNA inhibition mechanism which accounts for the cytotoxicity of many antibiotics. Both MRSA and P. aeruginosa, well noted for their insusceptibility to most antibiotics, were inhibited by all extracts especially by the roots, trunk and stem barks.

However, against the fungi, only the fruit (MID: 100 μg/spot) and leaf (MID: 400 μg/spot) extracts showed inhibition against C. herbarum. The results revealed that the G. atroviridis extracts were almost completely antibacterial and not antifungal. The strong antibacterial activity of all the extracts maybe attributed maybe to the presence of xanthones and related metabolites that have been implicated for the potent antibacterial activity in other species of Garcinia (Iinuma et al., 1996a,b).

All the extracts, except for the fruits, showed strong antioxidant activity (low absorbance values) by both the FTC and TBA methods. The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation. Subsequently, at a later stage of lipid oxida-
peroxide decomposes to form carbonyl compounds that are measured using the TBA method. The absorbance values of the fruit extract exceeded the negative controls (without extract) at the end point in both methods indicating the absence of antioxidant activity. However, the extracts of the remaining plant parts exhibited very strong antioxidant activity within the ranges of 64–90% by the FTC method and 87–93% by the TBA method, surpassing the activity of the standard commercial antioxidant, α-tocopherol (Fig. 1(a, b)). The pattern of activity was very similar for both methods. Among the extracts, the trunk bark showed the strongest antioxidant activity whereas the stem bark displayed the weakest activity. The difference in colour of the pigments found in the trunk and stem bark which are yellow and orange–red, respectively, serves as a preliminary indication of the variation in the major component(s) present in both plant parts. The antioxidant of the extracts was expected as it has been shown by xanthones isolated from other species of *Garcinia* (Minami et al., 1995, 1996).

Antitumour-promoting activity was measured as the ability of the extracts to inhibit the induction of EBV early antigen in Raji cells by the tumour-promoter PMA. Very strong antitumour-promoting activity was displayed by the trunk and stem bark extracts that inhibited EBV activation by about 90% (Table 2). Both the fruit and leaf extracts inhibited tumour-promotion in Raji cells by 67.5 and 64.7%, respectively. However, in an earlier report (Murakami et al., 1995), the fruit extract of *G. atrovirens* obtained in Thailand was found to be only weakly inhibitory towards EBV activation. Such differences in biological activity levels of crude plant extracts may result from climatic and soil variation (Evans, 1989).

The strong cytotoxicity of the root extract towards Raji cells (cell viability: 14.4%) at the test concentration of 200 μg/ml precluded the determination of antitumour-promoting activity. Except for the root extract, the other extracts were not cytotoxic towards Raji cells (cell viability: >95%) at the test concentration. The antitumour-promoting activity shown by the stem and trunk barks may be related to the antioxidant activity (Fig. 1) since oxidative-stress is closely associated with tumour promotion (Halliwell and Gutteridge, 1989).

Table 2

<table>
<thead>
<tr>
<th>Crude extract</th>
<th>% Inhibition (%)</th>
<th>% Cell viability</th>
</tr>
</thead>
<tbody>
<tr>
<td>(200 μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (untreated cells)</td>
<td>–</td>
<td>99.3</td>
</tr>
<tr>
<td>Fruits</td>
<td>67.5</td>
<td>97.3</td>
</tr>
<tr>
<td>Leaves</td>
<td>64.7</td>
<td>96.1</td>
</tr>
<tr>
<td>Stem bark</td>
<td>89.0</td>
<td>96.5</td>
</tr>
<tr>
<td>Trunk bark</td>
<td>90.9</td>
<td>96.4</td>
</tr>
<tr>
<td>Roots</td>
<td>n.d.*</td>
<td>14.4</td>
</tr>
</tbody>
</table>

* Not determined because the root extract was significantly cytotoxic towards Raji cells.
Again, xanthones and allied metabolites may be responsible for the antitumour-promoting activity.

None of the extracts exhibited cytotoxicity against CEM-SS cells (CD$_{50}$: $>30$ µg/ml), but only the root extract was mildly lethal (LC$_{50}$: 654.5 µg/ml) towards brine shrimp nauplii. The similar trend of non-toxicity in both the cell line and brine shrimp to assays is consistent with the strong correlation previously established between cytotoxicity and brine shrimp lethality (McLaughlin et al., 1991; Anderson et al., 1991). Therefore, at a preliminary stage it may be suggested that the *G. atroviridis* extracts possess non-toxic, antimicrobial, antitumour-promoting and antioxidant activities which renders them suitable as potential therapeutics. Differences in the profiles of biological activities of the various extracts suggest the presence of different constituents especially between the fruits and leaves with the other parts.

Acknowledgements

The authors wish to thank Universiti Putra Malaysia and the National Council for Research and Development (IRPA 03-02-04-0043 and 03-02-04-0045) for financial support, and Okayama University for a fellowship extended to M.M. Mackeen.

References


Screening of Zingiberaceae extracts for antimicrobial and antioxidant activities

M. Habsah a, M. Amran a, M.M. Mackeen a, N.H. Lajis a,*, H. Kikuzaki b, N. Nakatani b, A.A. Rahman c, Ghafar d, A.M. Ali e

a Department of Chemistry, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
b Department of Food and Nutrition, Faculty of Human Life Science, Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka, Japan
c Department of Biology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
d Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
e Department of Biotechnology, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Received 14 April 1999; received in revised form 22 June 1999; accepted 6 April 2000

Abstract

Dichloromethane and methanol extracts of 13 Zingiberaceae species from the Alpinia, Costus and Zingiber genera were screened for antimicrobial and antioxidant activities. The antimicrobial activity of most of the extracts was antibacterial with only the methanol extract of Costus discolor showing very potent antifungal activity against only Aspergillus ochraceous (MID, 15.6 μg per disc). All the extracts showed strong antioxidant activity comparable with or higher that of α-tocopherol. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Zingiberaceae; Antimicrobial activity; Antioxidant activity

1. Introduction

The Zingiberaceae is among the plant families which are widely distributed throughout the tropics particularly in Southeast Asia. In Peninsular Malaysia the Zingiberaceae are a component of the herbaceous ground flora of the rainforest. It is estimated that there are 150 species of ginger belonging to 23 genera found in Peninsular Malaysia (Holttum, 1950). Zingiberaceae species grow naturally in damp, shaded parts of the lowland or on hill slopes, as scattered plants or thickets. Most members of the family are easily recognised by the characteristic aromatic leaves and fleshy rhizome when both of them are crushed and also by the elliptic to elliptic–oblong leaves arranged in two ranks on the leaf-shoot.

In the Southeast Asian region, several species of Zingiberaceae are used as spices, medicines, flavouring agents and as the source of certain dyes (Burkill, 1966). Several species from the genera Alpinia, Anomum, Curcuma, Costus, Kaempferia and Zingiber are major ingredients in traditionally...
Table 1
List of plant species and yield of extracts

<table>
<thead>
<tr>
<th>Plant</th>
<th>Yield (w/w) (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Herbarium specimen No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alpinia hookeriana</em> Val.</td>
<td>0.34</td>
<td>AH-4/53</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td><em>Alpinia malaccensis</em> Rosc.</td>
<td>0.20</td>
<td>AML-5/34</td>
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<tr>
<td>CH₂Cl₂</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td><em>Alpinia mutica</em> Roxb.</td>
<td>0.46</td>
<td>AM-2/33/71</td>
</tr>
<tr>
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<td>0.64</td>
<td></td>
</tr>
<tr>
<td><em>Alpinia mutica</em> Roxb.</td>
<td>0.43</td>
<td>AN-3</td>
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<td>0.21</td>
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<tr>
<td><em>Alpinia rafflesiana</em> Wall.</td>
<td>0.36</td>
<td>AR-6</td>
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<tr>
<td>CH₂Cl₂</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td><em>Alpinia vitellina</em> (Lindl.) Ridl.</td>
<td>0.15</td>
<td>AV-1</td>
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<tr>
<td>CH₂Cl₂</td>
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<tr>
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<td>0.13</td>
<td>CD-8/10/12/63</td>
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<td>0.05</td>
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</tr>
<tr>
<td>MeOH</td>
<td>0.13</td>
<td>CM-11/13</td>
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<tr>
<td><em>Costus megalohactea</em> K. Schum.</td>
<td>0.14</td>
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<tr>
<td>MeOH</td>
<td>0.13</td>
<td></td>
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<tr>
<td><em>Costus villosissimus</em> Jacq.</td>
<td>0.03</td>
<td>CV-9</td>
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<td>0.09</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td></td>
<td>ZC-21/41</td>
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<td>0.14</td>
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</tr>
<tr>
<td>CH₂Cl₂</td>
<td>0.41</td>
<td>ZO-16/44</td>
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<tr>
<td>MeOH</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td><em>Zingiber macroglossum</em> Val.</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>0.37</td>
<td>ZM-22</td>
</tr>
<tr>
<td></td>
<td>0.48</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Weight (g) of crude extracts (CH₂Cl₂ and MeOH) per 100 g of dried plant material.

prepared tonics locally called ‘Jamu’, which are commercially available.

Although there are several reports on the constituents of the Zingiberaceae plants of Malaysia, these studies did not report the biological activities of the isolated compounds or crude extracts (Sirat, 1994; Sirat and Nordin, 1994; Sirat and Liamen, 1995; Sirat and Nordin, 1995; Sirat et al., 1994; Jamil and Sirat, 1996; Sirat et al., 1996). It is known that several species from the Zingiberaceae displayed antioxidant and antimicrobial activities (Iwu and Anyanwu, 1982; Bandara et al., 1989; Jitoe et al., 1992; Yamada et al., 1982; Haraguchi et al., 1996). In view of this, we screened the dichloromethane (less-polar) and methanol (polar) extracts of the 13 species of Zingiberaceae previously unstudied for antimicrobial and antioxidant activities, except for *Zingiber cassumunar*.

2. Materials and methods

2.1. Plant materials and preparation of extracts

All plant materials were collected from the nursery of the Germplasm Unit of Universiti Putra Malaysia (Table 1). Herbarium voucher specimens were prepared and deposited at the Department of Biology Herbarium, Universiti Putra Malaysia. Ahmad A. Rahman at the Department of Biology, Universiti Putra Malaysia, identified the specimens. About 300 g of the rhizome and root parts of the plant were cut into smaller pieces and air-dried under the shade. The materials were then successively extracted with dichloromethane and methanol thrice for each solvent. Extracts of each solvent were evaporated under reduced pressure and the final residues were used for the bioassays.

2.2. Microorganisms

The bacterial and fungal stock cultures were maintained on nutrient agar (NA) and potato dextrose agar (PDA) slants, respectively, which were stored at 4°C. For the purpose of antimicrobial evaluation, six microorganisms, i.e. *Bacillus substilis* (gram-positive), methicillin-resistant
Table 2
Minimum inhibitory dose (μg per disc) for antimicrobial activity of Zingiberaceae extracts

<table>
<thead>
<tr>
<th>Microorganism sample</th>
<th>B. subtilis</th>
<th>MRSA</th>
<th>P. aeruginosa</th>
<th>A. ochraceous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpinia hookeriana Val.</td>
<td>CH₂Cl₂</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Alpinia malaccensis Rosc.</td>
<td>CH₂Cl₂</td>
<td>1000</td>
<td>1000</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>1000</td>
<td>1000</td>
<td>250</td>
</tr>
<tr>
<td>Alpinia mutica Roxb.</td>
<td>CH₂Cl₂</td>
<td>125</td>
<td>1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Alpinia nutans Rosc.</td>
<td>CH₂Cl₂</td>
<td>250</td>
<td>1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Alpinia rafflesiana Wall.</td>
<td>CH₂Cl₂</td>
<td>250</td>
<td>1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>250</td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>Alpinia vitellina (Lindl.) Ridl.</td>
<td>CH₂Cl₂</td>
<td>1000</td>
<td>&gt;1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Costus discolor Rosc.</td>
<td>CH₂Cl₂</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Costus megalobractea K. Schum.</td>
<td>CH₂Cl₂</td>
<td>500</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Costus spiralis Rosc.</td>
<td>CH₂Cl₂</td>
<td>1000</td>
<td>1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Costus villosissimus Jacq.</td>
<td>CH₂Cl₂</td>
<td>1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Zingiber cassumunar Roxb.</td>
<td>CH₂Cl₂</td>
<td>250</td>
<td>&gt;1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Zingiber ottensii Val.</td>
<td>CH₂Cl₂</td>
<td>1000</td>
<td>&gt;1000</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Zingiber macroglossum Val.</td>
<td>CH₂Cl₂</td>
<td>250</td>
<td>1000</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>MeOH</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

* All extracts were inactive against *E. coli* and *C. albicans.*

*Staphylococcus aureus* (MRSA) (gram-positive), *Escherichia coli* (gram-negative), *Pseudomonas aeruginosa* (gram-negative), *Candida albicans* (fungus) and *Aspergillus ochraceous* (fungus), were
cultured in the appropriated broths at 30°C overnight. The concentrations of the cultures were adjusted using a spectrophotometer ($\lambda$, 600 nm) to $10^5$–$10^6$ colony forming units (CFU) per ml.

2.3. Antimicrobial activity assay

Agar cultures of the test microorganisms were prepared as described earlier (Mackeen et al., 1997). For initial screening, 1 mg of extract was loaded onto each Whatman No. 1 filter paper discs ($\varnothing$, 6 mm) and placed on the previously inoculated agar. The plates were inverted and incubated for 24 h at 30°C. Clear inhibition zones around the discs indicated the presence of antimicrobial activity. Extracts that showed positive activity in the preliminary screening were serially diluted (two-fold) and loaded on filter paper discs. These serially diluted doses of the extracts were assayed in triplicate as described above to determine the minimum inhibitory dose (MID), i.e. the minimum dose per disc to inhibit growth of the test microorganism.

2.4. Antioxidant activity assay

This assay was carried out as described in the modified method of Kikuzaki and Nakatani (1993). A mixture of 4 mg of a sample (final concentration, 0.02% w/v) in 4 ml of 99.5% ethanol, 4.1 ml of 2.5% linoleic acid in 99.5% ethanol, 8 ml of 0.02 M phosphate buffer (pH 7.0) and 3.9 ml of water contained in a screw-cap vial ($\varnothing$, 38 × 75 mm) was placed in an oven at 40°C in the dark. To 0.1 ml of this mixture, 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% ammonium thiocyanate was added. Three minutes after the addition of 0.1 ml of $2 \times 10^{-2}$ M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm every 24 h until 1 day after absorbance of the control reached its maximum value.

![Fig. 1. Antioxidant activity of Alpinia dichloromethane extracts.](Image)
3. Results and discussion

In general, most of the extracts evaluated for antimicrobial activity were active against bacteria and were almost completely inactive against fungi (Table 2). Furthermore, the antimicrobial activity of almost all of the dichloromethane extracts was much stronger than the methanol extracts. The dichloromethane extracts of *A. rafflesiana* showed the most broad-spectrum activity, i.e. towards *B. substilis*, MRSA, *P. aeruginosa* and *A. ochraceous*, followed by the dichloromethane extracts of *A. mutica*, *A. nutans* and *Z. macroglossum* against *B. substilis*, MRSA and *P. aeruginosa*. The most potent inhibitory activity of a dichloromethane extract was shown by *A. mutica* with the MID of 125 μg per disc against both *B. substilis* and MRSA.

The methanol extract of *C. discolor* displayed specific narrow-spectrum antifungal activity only against *A. ochraceous* at a very potent MID of 15.6 μg per disc. Other methanol extracts showing significant antimicrobial activity (MID: 250–500 μg per disc) were those of *A. malaccensis* and *A. rafflesiana*. All methanol and dichloromethane extracts failed to inhibit the growth of *E. coli* and *C. albicans*, and showed insignificant activity (weak or no activity) against MRSA.

Antioxidant activity was shown by both the dichloromethane and methanol extracts of all the Zingiberaceae species assayed (Figs. 1–4). The absorbance value of the untreated control approached a value of two on the 10th day. All the extracts showed similar levels of inhibitory activity towards lipid oxidation. The antioxidant activity of all the extracts was close to or higher than α-tocopherol. As in the antimicrobial activity results, some of the dichloromethane extracts showed stronger activity than the methanol extracts with reference to α-tocopherol namely the
extracts of *C. megalobractea*, *C. spiralis* (Fig. 3) and *Z. cassumunar* (Fig. 4). This observation suggests that less-polar component(s) present in the dichloromethane extracts contributed towards the increased activity over the methanol extracts. Some less-polar constituents such as curcuminoids, kava pyrones, and gingerols isolated from Zingiberaceous plants have been reported to possess biological activities such as antifungal, antioxidant, insecticidal, anti-inflammatory activities (Kikuzaki and Nakatani, 1993; Kikuzaki et al., 1994; Masuda and Jitoe, 1994; Tawata et al., 1996). Therefore, the significant antioxidant and antimicrobial properties of the Zingiberaceae extracts is particularly relevant to their use as a major ingredient in the preparation of 'Jamu'. Accordingly, this implies the inhibition of microbial pathogenesis, and cellular oxidation that is linked to pathological incidents such as heart disease, aging and cancer.

**Acknowledgements**

The authors wish to thank the Ministry of Science, Technology and the Environment for the fund provided under the Intensified Research in Priority Areas Research Grant (No. 09-02-04-0067). HM thanks Universiti Putra Malaysia for granting study leave from her teaching duties. N. Nakatani and H. Kikuzaki gratefully acknowledge the Programme for Promotion of Basic Research Activities for Innovative Biosciences.

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**Fig. 3.** Antioxidant activity of *Costus* extracts. (■), control (untreated); (♦), $\alpha$-tocopherol; (△), *C. discolor* (CH$_2$Cl$_2$); (+), *C. discolor* (MeOH); (▲), *C. megalobractea* (CH$_2$Cl$_2$); (□), *C. megalobractea* (MeOH); (●), *C. spiralis* (CH$_2$Cl$_2$); (●), *C. spiralis* (MeOH); (○), *C. eillosissimus* (CH$_2$Cl$_2$); (◇), *C. eillosissimus* (MeOH).
Fig. 4. Antioxidant activity of Zingiber extracts. (■), control (untreated); (○), α-tocopherol; (∆), Z. macroglossum (MeOH); (▲), Z. cassumunar (CH₂Cl₂); (●), Z. cassumunar (MeOH); (●), Z. macroglossum (CH₂Cl₂); (○), Z. ottensii (CH₂Cl₂); (◇), Z. macroglossum (MeOH).

References


Pharmacological study on antidepressant activity of 50% ethanol extract of a formulated ayurvedic product in rats

C.P. Bopaiah a, N. Pradhan a,*, B.S. Venkataram b

a Department of Psychopharmacology, National Institute of Mental Health and Neurosciences, Bangalore 560029, India
b Ayurvedic Research Unit, National Institute of Mental Health & Neurosciences, Bangalore 560029, India

Received 10 October 1999; received in revised form 10 March 2000; accepted 10 April 2000

Abstract

The effects of 50% ethanol extract of one formulated ayurvedic product, consisting of a mixture of medicinal plant species, was investigated on behavioral despair test (forced swimming test, FST), central dopaminergic and serotonergic activity in rats. The effects on the forced swimming test were assessed along with the levels of dopamine (DA), serotonin (5-HT) and its metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in striatum, frontal cortex, hippocampus, hypothalamus and brain stem after 21 days of chronic oral administration of the extract (500 and 1500 mg/kg-body weight). The extract significantly increased climbing behavior at 500 mg/kg and increased swimming behavior by reducing immobility time at 1500 mg/kg when compared with the control group in forced swimming test ($P < 0.05$). This showed that the active substances present in 50% ethanol extract of the ayurvedic preparation possess antidepressant activity and their specificity towards particular behavior, depends on the concentration of the extract. Further it showed that the enhancement of active behavior in FST is not due to generalized motor activity. The neurochemical estimations revealed the swim stressor inducing alterations in the levels of DA, 5-HT and their metabolites HVA and 5-HIAA in the brain regions assayed as compared with the non-stressed control rats. These changes were prevented in the extract treated rats. The 500 mg/kg extract treated group had significantly increased the levels of DA in frontal cortex, hypothalamus and hippocampus whereas the 5-HT in hypothalamus ($P < 0.05$). However, there were no significant changes in the levels of HVA and 5-HIAA. These behavioral and biochemical results indicate antidepressant properties of the extract, which may be mediated by the dopaminergic and serotonergic mechanisms in rat brain. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Ayurveda; Forced swimming test; Dopamine; Serotonin; 5-Hydroxyindoleacetic acid; Homovanillic acid

1. Introduction

A number of singles and compound drug formulations of plant origin are mentioned in Ayurveda, the ancient Indian traditional system of medicine for the treatment of psychiatric disor-
ders (Charak Samhita, 1941). These natural plant products are used in different forms like powders, decoctions, tinctures, etc. with encouraging results. There are some preparations recommended in ayurvedic classics in the treatment of manovikara (psychiatric disorders) with clinical features of depressive illness (Charak Samhita, 1941). In ayurvedic practice, the compound preparations are found to be more effective than a single drug treatment. In this study, the 50% ethanol extract of one such ayurvedic product recommended by ayurvedic physician for the treatment of depression was evaluated for antidepressant activity (personal communication). The constituents of the formulation, with ayurvedic nomenclature, part of the plant used and the percent composition of the ingredients are given in parenthesis: (1) Acorus calamus Linn. (Araceae) (vacha, rhizome, 8%), (2) Nardostachys DC. (Valerianaceae) (jatamansi, root, 25%), (3) Piper longum Linn. (Piperaceae) (pippali, fruit, 10%) (4) Plumbago zeylanica Linn. (Plumbaginaceae) (chitraka, root, 10%), (5) Withania somnifera Linn. (Solanaceae) (ashwaganda, root, 35%) and (6) Allium sativum Linn. (Liliaceae) (lasuna, bulb, 12%).

So far there are no pharmacological evidences to demonstrate the antidepressant properties and its mechanism of action. However, there are reports to show that one of the ingredients of this formulation, W. somnifera possessing anti-stress activity (Archana and Namasivayam, 1999). Also, there are studies to show the sedative and tranquilizing effects of A. calamus and Nardostachys (Arora, 1965; Vohora et al., 1990; Houghton, 1999). Animal study has showed that the chronic treatment of Nardostachys jatamansi significantly increases the levels of biogenic amines and inhibitory amino acids (Prabu et al., 1994).

In the laboratory the acute study showed that 50% ethanol extract of this ayurvedic preparation possessing anti-stress activity in the animal model of depression (Porsolt et al., 1978a). The effectiveness of the extract was found at two optimal doses (500 and 1500 mg/kg-body weight). Further the extract did not show any toxic symptoms up to 5 g/kg when administered orally (data’s are not published). However, the chronic treatment of antidepressant drugs are found to be more effective in alleviating depression (Borsini and Meli, 1988).

The forced swimming test (FST) is a behavioral test widely used to screen new potent antidepressant drugs in rats and mice (Porsolt et al., 1978a, 1979). This test is sensitive and specific to all major classes of antidepressant drugs including tricyclic antidepressants, serotonin specific reuptake inhibitors and monoamine oxidase inhibitors (Borsini and Meli, 1988; Detke et al., 1995). However, drugs enhancing motor activity may give ‘false’ positive effect in FST (Porsolt et al., 1978a). Further there are reports to show that drugs reducing motor activity may exert anti-immobility effect (Porsolt et al., 1978a; Borsini et al., 1985).

There are extensive evidences to show that different types of experimental stressors induce neurochemical and hormonal changes in animal models of depression (Katz et al., 1981; Anisman and Zacharko, 1990; Willner, 1991). The forced swimming test in rats and mice induces alterations in the levels of monoamine and indoleamines, which are reminiscent to human symptoms of depression. It also shows that this swim-stressor modulates the neurotransmitter changes in a regionally specific manner (Miura et al., 1993; Kirby et al., 1995). There are reports to show that the antidepressant drugs are effective in preventing the behavioral and biochemical changes induced by FST (Miura et al., 1993; Imperato et al., 1994).

The behavioral changes in FST, locomotor activity and levels of dopamine (DA), serotonin (5-HT) and its metabolites homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in striatum, hippocampus, hypothalamus, frontal cortex and brain stem were used as parameters for evaluating antidepressant activity of the ayurvedic product in rats.

2. Materials and methods

2.1. Plant material and extract preparation

Pharmacognostically identified dried parts of individual plant ingredients were collected from
the local market and authenticated by Dr S.N. Yoganarasimhan from the Regional Research Center for Herbarium and Crude Drug Museum, Bangalore, India (RRCBI). The herbarium voucher specimen numbers are (1) *A. calamus* L. (RRCBI-373) (2) *Nardostachys* DC. (RRCBI-120) (3) *P. longum* L. (RRCBI-232) (4) *P. zeylanica* L. (RRCBI-3142) (5) *W. somnifera* L. (RRCBI-788) and (6) *A. sativum* L. (RRCBI-636).

They were coarsely powdered and the composition was formed. The composition was extracted with water ethanol 50:50 using Soxhlet extractor. Then the extract was concentrated on a water bath under reduced pressure. The semisolid form of the extract was dried in a vacuum dissector over anhydrous calcium chloride. Yield was 1.6% (w/w). The suspension of the extract (0.5 ml) was prepared using Tween 80:water (1:9) at concentrations of 500 and 1500 mg:kg-body weight and administered orally. The control group received the same volume of the vehicle.

2.2. Experimental design

2.2.1. Animals

Adult male, Sprague–Dawley rats weighing 200–230 g obtained from the Central Animal Research Facility of the National Institute of Mental Health and Neuroscience, Bangalore, India, were used for the experiment. The rats were fed with standard Hindustan Lever chow pellets and water ad lib. They were housed four per cage and reared in a 12/12-h light/dark cycle (lights on at 07:00 h) temperature controlled (23 ± 1°C) animal house.

Three groups (*n* = 8, for FST and *n* = 10, for locomotor activity) of rats were randomly selected for behavioral experiments. Group 1 was taken as control while, the remaining two groups were administered with extract at doses of 500 and 1500 mg/kg-body weight, respectively. The suspension of the extract was orally administered for 21 days. After 90 min of last dosage on 21st day, the rats were subjected to swim-stress for 30 min. Then they were decapitated and the tissue samples (striatum, hippocampus, frontal cortex, hypothalamus and brain stem) were collected for assay. The extract was administered between 08:00 and 09:00 h and the experiment was performed between 09:00 and 13:00 h to reduce the confounding influence of diurnal variations.

2.3. FST

The FST is commonly used for both behavioral and biochemical experiments. For behavioral experiment the procedure used was the modification to that described by Porsolt et al. (1978a, 1979). In this study, the rats were exposed to forced swimming only once without pretest session. On the test day, 90 min after the extract or vehicle treatment, individual rats from each group were dropped into a glass cylinder (height, 45 cm; diameter, 22 cm) containing tap water of depth 35 cm and the temperature maintained at 35–37°C. During this period the rat exhibits a distinct types of active behavior. Time sampling technique was used to score the three types of active behavior (immobility, swimming and climbing) as described by Detke et al. (1995). The test session lasted for 8 min. At the end of each 5-s period, the scorer, who is unaware of the treatment, recorded one of the following behaviors using a stopwatch. (1) Immobility — a rat was judged to be immobile when it remained floating in the water without struggling and was making only those movements necessary to keep its head above water; (2) swimming — if the rat makes active swimming motions, more than necessary to merely maintain its head above water; (3) climbing — a rat judged to be climbing when it was making active movements with its forepaws in and out of the water, usually directed against the walls. A 3-min period was allowed for acclimatization and last 5-min period scoring was taken for analysis. Data is expressed in number of 5 s periods spent in each category of behavior (score).
For biochemical experiment the rats was forced to swim for 30 min in the same conditions after 90 min of last dosage on 21st day. Then they were sacrificed and tissue samples were collected for assay.

2.4. Locomotor activity

The motility of animals was measured using a horizontal 2D-Optovarimax monitoring cage (Columbus Instruments International, OH). It consists of two arrays of 15 infrared beams, which are placed perpendicular to each other. The beams are spaced 25.4 mm apart and each beam is very narrow (3 mm in diameter). Each interruption of a beam generates an electric impulse, which is processed at a sampling frequency of 10 Hz by an interfaced microprocessor. The ambulatory activity is a record of the distance traveled in centimeters and ambulatory time in seconds. The record is obtained when beams outside the box surrounding the animal are interrupted. The beam in all directions immediately surrounding the animal is defined as the box.

Animals were acclimatized to the monitoring cage for 15 min/day before the start of behavioral experiments. The monitoring began 5 min after the rat was placed in the monitoring cage on the day of the experiment. The session duration for the measurement of ambulatory behavior was 30 min. The experiment was carried out in a sound proof room between 09:00 and 13:00 h to reduce the confounding influence of diurnal variation in motility.

2.5. Biochemical analysis

Rats from each group were sacrificed between 09:00 and 12:00 h to reduce the influence of diurnal variations. The brain was dissected and the tissue samples was separated on ice and homogenized in ice cold 0.1 M perchloric acid with an internal standard (Isoproterenol) at a concentration of 100 ng/ml. The homogenates were centrifuged at 10 000 × g, the supernatant was separated and ultrafiltered through an Amicon micropartition system (MPS). The clear ultrafiltrate was used for quantification of DA, 5-HT and their metabolites HVA and 5-HIAA by High Performance Liquid Chromatography (HPLC) with electrochemical detection (Pradhan et al., 1990). An isocratic HPLC system comprised of a HPLC pump (Model 302 Gilson, France) with a pulse damper, a Rheodyne injector with a 100 µl loop (rheodyne Corp.), an Amperometric detector (LC 4B Bioanalytic System), an on-line solvent filter, pre filters and C-18 guard columns (Supelco). The glassy carbon electrode potential was set to 0.70 V for DA, 5-HT, HVA and 5-HIAA. The compounds were eluted with triethyl amine phosphate (TEA) buffer system (pH 2.80) containing 0.1 mM EDTA and 50 mM/l of heptane sulphonic acid as an ion-pairing agent on a 5 cm C-18, 3-µm packing (Supelco) column with a flow rate of 0.8 ml/min at ambient temperature.

2.6. Statistics

The behavioral and biochemical data was analyzed using a statistical package (SPSS: release 6) on Pentium II–233 MHz computer. Means and standard error of means (± S.E.M.) were calculated for all the parameters. Comparisons between the various groups were made using a one-way analysis of variance. Post-hoc comparisons were performed using Tukey’s Honestly Significant Difference test using 5% level of significance.

3. Results

3.1. FST

The behavioral scores of control and extract treated groups are shown in Table 1. One-way ANOVA showed that there is a significant reduction in the immobility time \(F(2,21) = 0.024, P < 0.05\), and increase in swimming \(F(2,21) = 0.00, P < 0.01\) and climbing behavior \(F(2,21) = 0.009, P < 0.05\). Post-hoc comparison test showed a significant difference in immobility and swimming behaviors at 1500 mg/kg and climbing behavior at 500 mg/kg-extract treated group when compared with the control group.
3.2. Locomotor activity

The ambulatory scores of control and extract treated groups are shown in Table 2. One way ANOVA showed that there is no change in the ambulatory behavior \( F(3,28) = 0.300, P > 0.05 \). The extract treated groups traversed less than control group.

3.3. Neurochemical parameters

The effects of swim-stress and extract treatment on the levels of DA, 5-HT, 5-HIAA and HVA in five different regions of brain are shown in Table 3.

3.3.1. DA levels

One-way ANOVA showed that there is a significant difference in the levels of DA between groups in striatum \( F(3,28) = 16.303, P < 0.01 \), hippocampus \( F(3,28) = 3.741, P < 0.05 \), frontal cortex \( F(3,28) = 6.320, P < 0.05 \) and hypothalamus \( F(3,28) = 6.125, P < 0.05 \). But in brain stem there was no significant difference between groups \( F(3,28) = 0.789, P > 0.05 \). In striatum the swim stressed control group showed significant increase in DA level when compared to non-stressed and 500 mg/kg extract treated group. However, 1500 mg/kg group showed higher level than remaining groups. In frontal cortex and hippocampus there was a significant increase at 500 mg/kg in comparison to swim-stressed control group. In hypothalamus the 500 mg/kg extract treated group showed significantly higher level of DA in comparison to both non-stressed and swim-stressed control groups.

3.3.2. HVA levels

One-way ANOVA showed that there is no significant difference in the levels of HVA between groups in striatum \( F(3,28) = 1.301, P > 0.05 \), hippocampus \( F(3,28) = 0.208, P > 0.05 \), frontal cortex \( F(3,28) = 1.482, P > 0.05 \), hypothalamus \( F(3,28) = 1.459, P > 0.05 \) and brain stem \( F(3,28) = 0.313, P > 0.05 \).

3.3.3. 5-HT levels

One-way ANOVA indicated a significant difference between groups in hypothalamus \( F(3,28) = 7.094, P < 0.05 \). Post-hoc comparison showed significant difference between swim-stressed control group and 500 mg/kg-extract treated group. However, there was no significant difference between groups in striatum \( F(3,28) = 2.208, P > 0.05 \), hippocampus \( F(3,28) = 0.926, P > 0.05 \), frontal cortex \( F(3,28) = 1.393, P > 0.05 \) and brain stem \( F(3,28) = 1.891, P > 0.05 \). But 500 mg/kg extract treated group showed higher level of 5-HT in all the regions when compared to other groups.

Table 1
Mean ± S.E.M. scores of immobility, swimming and climbing in FST of control and extracted treated groups \((n = 10)\)

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Dosage (mg/kg)</th>
<th>Immobility</th>
<th>Swimming</th>
<th>Climbing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>44.0 ± 1.79</td>
<td>11.87 ± 1.21</td>
<td>4.12 ± 1.09</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>41.37 ± 2.24</td>
<td>8.12 ± 0.76</td>
<td>10.62 ± 1.44*</td>
</tr>
<tr>
<td>3</td>
<td>1500</td>
<td>36.00 ± 1.7*</td>
<td>17.62 ± 4.9*</td>
<td>6.37 ± 1.43</td>
</tr>
</tbody>
</table>

* Significantly different from control group \((P<0.05)\).

Table 2
Mean ± S.E.M. ambulatory scores of control and extract treated groups \((n = 10)\)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (Group-1)</th>
<th>500 mg/kg (Group-2)</th>
<th>1500 mg/kg (Group-3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total time spent in ambulatory behavior(s)</td>
<td>93.320 ± 18.38</td>
<td>86.950 ± 7.34</td>
<td>78.230 ± 10.39</td>
</tr>
</tbody>
</table>
Table 3
Mean ± S.E.M. concentrations (in ng/g wet weight of brain tissue) of DA, HVA, 5-HT and 5-HIAA in the striatum, hippocampus, frontal cortex, hypothalamus and brain stem of normal, swim-stressed and extract treated rats (n = 8)

<table>
<thead>
<tr>
<th>Variable</th>
<th>NSC (Group-1)</th>
<th>SSC (Group-2)</th>
<th>500 mg/kg (Group-3)</th>
<th>1500 mg/kg (Group-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>1573 ± 305</td>
<td>9931 ± 1248*</td>
<td>3479 ± 477**</td>
<td>10336 ± 17*,***</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>776 ± 235</td>
<td>85.13 ± 22.5</td>
<td>1489 ± 465**</td>
<td>118 ± 71***</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>579 ± 244</td>
<td>18.25 ± 4.2</td>
<td>1313 ± 345**</td>
<td>1197 ± 450</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>727 ± 197</td>
<td>328 ± 61</td>
<td>1822 ± 42*,**</td>
<td>564 ± 244*,***</td>
</tr>
<tr>
<td>Brain stem</td>
<td>375 ± 106</td>
<td>926 ± 342</td>
<td>771 ± 211</td>
<td>686 ± 310</td>
</tr>
<tr>
<td><strong>HVA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>1260 ± 345</td>
<td>2811 ± 660</td>
<td>1999 ± 664</td>
<td>2708 ± 772</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>663 ± 125</td>
<td>1285 ± 374</td>
<td>925 ± 177</td>
<td>1357 ± 309</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>886 ± 276</td>
<td>758 ± 218</td>
<td>648 ± 213</td>
<td>773 ± 109</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>665 ± 99</td>
<td>1463 ± 522</td>
<td>1010 ± 197</td>
<td>1325 ± 157</td>
</tr>
<tr>
<td>Brain stem</td>
<td>485 ± 45</td>
<td>585 ± 80</td>
<td>569 ± 91</td>
<td>563 ± 93</td>
</tr>
<tr>
<td><strong>5-HT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>572 ± 84</td>
<td>1314 ± 369</td>
<td>1647 ± 180</td>
<td>1550 ± 502</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>886 ± 138</td>
<td>426 ± 113</td>
<td>815 ± 256</td>
<td>780 ± 153</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>370 ± 60</td>
<td>360 ± 57</td>
<td>451 ± 116</td>
<td>267 ± 61</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1566 ± 261</td>
<td>429 ± 66*</td>
<td>1757 ± 327**</td>
<td>791 ± 185***</td>
</tr>
<tr>
<td>Brain stem</td>
<td>999 ± 119</td>
<td>464 ± 108</td>
<td>951 ± 299</td>
<td>861 ± 963</td>
</tr>
<tr>
<td><strong>5-HIAA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striatum</td>
<td>375 ± 54</td>
<td>520 ± 44</td>
<td>810 ± 117*</td>
<td>558 ± 125</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>482 ± 146</td>
<td>402 ± 67</td>
<td>503 ± 175</td>
<td>502 ± 88</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>291 ± 46</td>
<td>461 ± 128</td>
<td>522 ± 220</td>
<td>489 ± 181</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>1879 ± 670</td>
<td>349 ± 78*</td>
<td>1297 ± 304</td>
<td>502 ± 108</td>
</tr>
<tr>
<td>Brain stem</td>
<td>1489 ± 336</td>
<td>465 ± 143</td>
<td>1426 ± 402</td>
<td>916 ± 152</td>
</tr>
</tbody>
</table>

* Significantly different from non-stressed control group (NSC).
** Significantly different from stressed control group (SSC group).
*** Significantly different from 500mg/kg treated group.

3.3.4. 5-HIAA levels
One-way ANOVA showed a significant difference in striatum and hypothalamus ($F(3,28) = 3.781, P < 0.05$) and ($F(3,28) = 3.632, P < 0.05$). In striatum, the 500 mg/kg group showed significantly higher levels when compared to non-stressed control group and in hypothalamus the swim-stressed control group showed significantly lower than non-stressed control group ($P < 0.05$). However, there was no significant difference in the levels of 5-HIAA between groups in hippocampus ($F(3,28) = 0.420, P > 0.05$), frontal cortex ($F(3,28) = 0.141, P > 0.05$) and brain stem ($F(3,28) = 2.894, P > 0.05$).

4. Discussion
The results of this study showed that chronic treatment of 50% ethanol extract of the ayurvedic preparation are effective in eliciting the active behavior in forced swimming test, central serotonergic and dopaminergic systems in rats. FST in rats or mice is widely used as an animal model of...
depression to screen new potent antidepressant drugs (Porsolt et al., 1977b, 1978a, 1979). This test is sensitive and specific to all major classes of antidepressant drugs including tricyclics, serotonin specific reuptake inhibitors and monoamine oxidase inhibitors (Borsini and Meli, 1988; Detke et al., 1995). The characteristic behavior scored in this test is termed as immobility, swimming and climbing. Antidepressant drug reduces immobility time, increases swimming and climbing behavior, depending on the concentration and the type of antidepressant drug administered.

The chronic treatment of the extract significantly reduced the immobility time and increased the swimming behavior at high dosage (1500 mg/kg). Whereas the climbing behavior were significantly high at low dosage (500 mg/kg). This shows that the 50% ethanol extract of the ayurvedic product possesses antidepressant activity and its specificity towards particular behavior may depend on the concentration of the extract. There are reports to indicate that immobility, swimming and climbing behaviors are enhanced by different groups of antidepressant drugs (Detke et al., 1995). The NE-selective uptake inhibitors like desipramine (DMI) and maprotiline (MAP) enhances the climbing behavior where as the serotonin specific reuptake inhibitors (SSRIs) like fluoxetine (FLX), sertraline (SRT) and paroxetine (PRX) enhance swimming but not climbing behavior. However, both the types of antidepressants reduce immobility behavior. Also recent studies show that the dopaminergic activation is also involved in struggling (climbing) behavior (David et al., 1981; Imperato et al., 1994; Cabib and Puglisi-Allegra, 1996). This implies that 50% ethanolic extract of the formulated ayurvedic preparation contain active chemical constituents contributed by the individual ingredients to elicit specific types of behavior in FST through noradergic, serotonergic and dopaminergic systems and thereby acting as an antidepressant agent. It also suggests that the activation of these systems may depend on the concentration of the extract. Further, there was no remarkable change in the ambulatory behavior on chronic treatment. The ambulatory behavior decreased in comparison to the control group but no significant difference was found. This ensures that any increases in mobility observed in the FST, after chronic treatments was not due to generalized locomotor effects.

The biochemical data suggest that the extract might mediate their antidepressant action through dopaminergic and serotonergic systems. The result showed that swim-stress for 30 min altered the levels of DA, 5-HT and their metabolites HVA and 5-HIAA in different functional regions of the rat brain. Chronic treatment of the extract was effective in preventing the swim-stress-induced alterations of neurotransmitters. There are substantial reports to indicate that stressors induce neurochemical and hormonal alterations, which are reminiscent of those, observed in depressed patients (Katz et al., 1981; Anisman and Zacharko, 1990; Willner, 1991). Clinically used antidepressant drugs are effective in blocking the stress induced neurochemical changes (Miura et al., 1993; Imperato et al., 1994; Kirby et al., 1995; Cabib and Puglisi-Allegra, 1996).

The swim-stressed control group showed marked increase in the levels of DA in striatum and brain stem whereas it decreased in frontal cortex, hippocampus and hypothalamus when compared to non-stressed control group. The HVA, metabolite of DA marginally enhanced in striatum, frontal cortex and hypothalamus and showed no remarkable changes in hippocampus and brain stem. On the other hand 5-HT level increased in striatum and remarkably reduced in frontal cortex, hypothalamus ($P < 0.05$) and brain stem without effecting hippocampus. Swim-stress reduced the level of 5-HIAA in hypothalamus and brain stem without changes in hippocampus and frontal cortex when compared with the non-stressed control group.

There are discrepancies in reporting the swim-stress-induced neurochemical changes in different regions of the brain. A recent report shows an increase in the levels of DA in striatum and 5-HT levels in frontal cortex and hypothalamus due to swim stress (Connor et al., 1999). Other studies found reduced striatal DA content and no significant changes in 5-HT levels in frontal cortex and hypothalamus due to swim-stress (Rossetti et al., 1993; Kirby et al., 1995). Further, Miura et al.
(1996) reported significant increases in DA, 5-HT and their metabolites in mice brain following swim-stress. The results showed an increase in the level of DA in striatum without affecting the 5-HT levels in hypothalamus. The variations in stress induced neurochemical changes depends on the experimental conditions adopted by different experimenters. As Kirby et al. (1997) pointed out, alterations induced by stressors depend on the intensity of stress induced and the techniques used for the assay of neurotransmitters.

The chronic treatment of the extract significantly enhanced the levels of DA in hippocampus, frontal cortex, hypothalamus and significantly reduced in striatum at 500 mg/kg when compared with the swim-stress control group. However, striatum showed significant increase at 1500 mg/kg when compared with non-stressed control group, but there were no remarkable changes in the content of DA in brain stem. The HVA levels did not show any significant differences between groups in all the regions assayed. This shows that the chronic extract treatment is effective in preventing the swim-stress-induced alterations of dopaminergic systems. It also indicates that the specificity of the extract are more pronounced towards hippocampus, hypothalamus and frontal cortex. Further it showed that the effectiveness of the extract are at low dose (500 mg/kg) and this may indicate that the significant increase in the climbing behavior of FST at 500 mg/kg was mediated through dopaminergic systems of frontal cortex, hypothalamus and hippocampus since there are reports to show that the struggling behavior in FST is enhanced through dopaminergic systems by antidepressant drugs (Cabib and Puglisi-Allegra, 1996).

The chronic extract treatment also prevented the alterations in 5-HT and 5-HIAA levels induced by swim-stress. It showed significant increase in the 5-HT and 5-HIAA levels in hypothalamus (P < 0.05) but higher levels were observed in striatum, frontal cortex, hippocampus and brain stem at 500 mg/kg when compared with the stress-control group. This shows that the extract is more specific towards hypothalamus serotonin system. However, it is difficult to ascertain that the immobility and swimming behavior is being mediated through serotonergic systems as the significance was seen only in hypothalamus at 500 mg/kg treated group and the active behaviors (immobility and swimming) were found to be effective at 1500 mg/kg group. So, the biochemical assay indicates that the potency of antidepressant activity is at low dosage (500 mg/kg). Further it indicates the mechanism of antidepressant action being mediated through both dopaminergic and serotonergic systems particularly through the DA systems of frontal cortex, hypothalamus and hippocampus whereas the 5-HT system in hypothalamus.

In conclusion, the chronic treatment of 50% ethanol extract of the formulated ayurvedic preparation are effective in FST and in preventing the stress induced by swim-stressor thereby, acting as antidepressant agents. Thus the present study permitted one to detect the antidepressant activity in 50% ethanol extract of the ayurvedic product on chronic treatment.

References


Charak Samhita, 1941. Nirnaya Sagar Press, Bombay, India.


Antidiarrhoeal and antiulcerogenic effects of methanolic extract of *Asparagus pubescens* root in rats

Paul A. Nwafor a,*, F.K. Okwuasaba b, L.G. Binda a

a Department of Pharmacology, College of Medical Sciences, University of Maiduguri, P.M.B. 1069 Maiduguri, Nigeria
b Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmaceutical Science, University of Jos., P.M.B 2084 Jos, Nigeria

Received 7 May 1999; received in revised form 8 November 1999; accepted 28 April 2000

Abstract

The effect of methanolic extract of *Asparagus pubescens* root on experimentally-induced diarrhoea and ulceration was investigated in rats. The extract (500–1500 mg/kg) dose-dependently, reduced significantly the intestinal propulsive movement, castor oil-induced diarrhoea and intestinal fluid accumulation. Yohimbine an α2-adrenoceptor blocker attenuated the antidiarrhoeal effect of the extract. The extract also reduced the ulcer indices induced by indomethacin and ethanol in a dose-related manner. The results indicate that its antidiarrhoeal and antiulcerogenic effects might in part be due to its α2-adrenoceptor stimulation and its active constituents respectively. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Asparagus pubescens* root; Antidiarrhoeal; Antiulcer; Methanolic extract

1. Introduction

*Asparagus pubescens* root (Liliaceae) is widely used in the north central part of Nigeria for treatment of diarrhoea and peptic ulceration. There is, however, no reference in literature as to the possible antidiarrhoeal and antiulcerogenic efficacy of the root either in human or animal studies. A search in NAPRALERT database did not yield such information (R. Quimby, University of Illinois, Chicago, personal communication, 1996). The present study was to establish if the root possesses any true antidiarrhoeal and antiulcerogenic properties.

2. Materials and methods

2.1. Preparation of extracts

The plant material used in this study was collected from Rukuba village in Jos metropolis (Plateau state) between April and September, 1995. The plant was identified and authenticated by Dr S.S. Sanusi, Department of Botany, University of Maiduguri. Specimen vouchers (FPS. 014) were made and deposited at the herbarium of...
the Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmaceutical Sciences, University of Jos. The dried root was pulverized by grinding using pestle an mortar. Then 57 g of the ground root was subjected to exhaustive soxhlet extraction in methanol (250 ml) for 72 h at 60°C. This gave a mean yield of 16.6 ± 0.23 g w/w of extract. The extract was stored at −4°C from where it was used when required.

2.2. Animal stock

Adult albino rats weighing 160–200 g were used in this study. All the animals were housed in a cross-ventilated room (temperature 22 ± 2.5°C, 12 h light/12 h dark cycle) and were fed with standard mash (Feedex Nig., Kaduna, Nigeria) and water ad-libitum.

2.3. Small intestinal propulsion

The effect of extract on intestinal propulsion in unanaesthetized rats was tested using the charcoal method of Capasso et al. (1976). They were fasted for 24 h but allowed free access to water. They were randomized and placed in six cages of five animals per cage. Group 1 was administered with normal saline (p.o) using orogastric cannula. Groups 2–4 were pretreated with *A. pubescens* extract 500–1500 mg/kg (p.o), respectively. Group 5 was pretreated with 100 μg/kg atropine (p.o) while group 6 received 1.0 mg/kg yohimbine (s.c) 10 min after 1500 mg/kg extract (p.o) was administered. After 1 h, each rat was administered with 1 ml charcoal meal (5% activated charcoal suspended in 10% aqueous tragacant), orally. The rats were killed 30 min later by cervical dislocation and bled, and the small intestine rapidly dissected out and placed on a clean surface. The small intestine was carefully inspected and the distance traversed by the charcoal meal from the pylorus was measured. The length of the whole small intestine was also measured. The distance traversed by the charcoal meal from the pylorus was expressed as a percentage of the distance from the pylorus to the ileocaecal junction.

\[
\text{Intestinal propulsion\%} = \frac{\ \text{Distance moved by the suspended charcoal head}}{\ \text{Whole length of small intestine}} \times 100 \%
\]

2.4. Castor oil-induced diarrhoea

A modification of the method of Awouters et al. (1978) by Nwodo and Alumanah (1991) was adopted. The rats were fasted for 24 h but allowed free access to water. They were randomized and placed in cages of five rats per cage. Group 1 was administered with normal saline. Groups 2 and 3 were given 500 and 1500 mg/kg of extract (p.o), respectively. Group 4 was administered (p.o) with diphenoxylate 5.0 mg/kg, group 5 was administered with 1.0 mg/kg yohimbine (s.c) and 10 min later, 1500 mg/kg of extract was given orally to both groups. After 1 h, each rat received 2 ml castor oil (p.o) and was observed for consistency of faecal matter and the frequency of defaecation for 3 h. Faeces were allowed to collect beneath the slit wire guazed cages. The wet faecal matters were easily read at the end of the experiment by lifting off the upper part of the cage containing the slit wire guaze and the animals.

2.5. Castor oil-induced fluid accumulation

This was determined according to the method of Robert et al. (1976) modified by DiCarlo et al. (1994). The rats were fasted for 24 h but allowed free access to water. They were randomized and placed in four cages of five rats each. All drugs were orally given. Group 1 was administered with normal saline. Group 2 was administered with castor oil. Groups 3 and 4 were pretreated with the extract 500 and 1500 mg/kg, respectively, 1 h prior to castor oil administration to all the rats. After 30 min, the rats were killed by cervical dislocation and exsanguinated, the small intestine was ligated at both pyloric sphincter and at the ileocaecal junctions. The entire small intestine was dissected out, its contents were expelled into a
graduated measuring cylinder and the volume of the contents was recorded.

2.6. Indomethacin-induced gastric ulceration

Pilot tests aimed at determining the effective dose of indomethacin required to produce reliable acute gastric ulceration in rats were done. This was achieved by administering varying doses of indomethacin (40, 60, and 100 mg/kg; Brussels, Belgium) to rats. In this way, the least effective dose (p.o) of indomethacin that produced 100% gastric ulceration was obtained. The dose was repeated to verify if the degree of ulceration will be reproducible. From these tests, 100 mg/kg produced gastric ulceration in all rats in 4 h.

The rats were randomized and divided into four groups of five rats each. Food was withdrawn 24 h and water 2 h before the commencement of experiment (Alphin and Ward, 1967). Group 1 was administered with 100 mg/kg indomethacin (p.o). Groups 2–4 were pretreated with 500–1500 mg/kg of extract, 1 h prior to administration of 100 mg/kg of indomethacin. The drugs were administered intragastrically via the aid of an orogastric cannula. Four hours later, the animals were killed by cervical dislocation. The stomachs were removed and opened along the greater curvature. The tissues were fixed with 10% formaldehyde in saline. Macroscopic examination was carried out with a hand lens and scored for the presence of lesions using Alphin and Ward (1967) method modified by Evbuonwa and Bolarinwa (1990). Ulcer index (UI) and preventive ratio of each of the groups pretreated with extract were calculated using the methods of Zaidi and Mukeriji (1958) and Nwafor et al. (1996).

\[
\text{UI} = \frac{\text{degree of ulceration} \times \text{percentage of group ulcerated}}{100}
\]

Preventive ratio

\[
= \frac{\text{UI(Ulcerated group} - \text{protected group)}}{\text{UI(Ulcerated group)}} \times 100
\]

Degree of ulceration = \[
\frac{\text{Total ulcer score}}{\text{No. of animals ulcerated}}
\]

2.7. Ethanol-induced gastric ulceration

The above procedure (as in indomethacin-induced ulceration) was repeated using ethanol (BDH) as an ulcerogen and the scoring method of Barry et al. (1988) was adapted.

2.8. Statistical methods

Results were expressed as the mean value ± S.E.M. and significance was determined by Students t-test. A probability level of less than 5% was considered significant.

3. Results

3.1. Small intestinal propulsion

In control animals (saline treated rats), the charcoal meal traversed 75.06% of the total length of the small intestine (Table 1). 500 mg/kg of extract, decreased the intestinal propulsion by 63.23% which is equivalent to 36.77% intestinal propulsive inhibition relative to control (P < 0.001). Similarly 1000 and 1500 mg/kg of extract, caused increase in intestinal propulsive inhibition of 51.15 and 61.68%, respectively. When 1500 mg/kg of extract was repeated in the presence of yohimbine (1.0 mg/kg), an \(\alpha_2\)-blocker, the intestinal propulsive inhibition decreased 40.17%. Atropine, an anticholinergic drug, caused intestinal propulsive decrease of 35.38% with a maximal intestinal inhibition of 64.62%.

3.2. Castor oil-induced diarrhoea

\(A. \text{pubescens}\) extract at the dose range of 500–1500 mg/kg caused a dose-dependent decrease in the number of faecal matters passed by the rats. A standard antidiarrhoeal drug (diphenoxylate) in the presence of extract (1500 mg/kg) inhibited the castor oil-induced diarrhoea by 83.58% (Table 2). However, in the presence of yohimbine, the antidiarrhoeal effect of the extract was reduced to 29.11%.
Table 1
Effects of *Asparagus pubescens* extract on intestinal propulsion in rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Mean intestinal length (cm)</th>
<th>Mean distance moved by charcoal (cm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml saline</td>
<td>81.40 ± 1.6</td>
<td>61.1 ± 1.5</td>
<td>24.94</td>
</tr>
<tr>
<td>500</td>
<td>82.4 ± 6.8</td>
<td>52.1 ± 1.6**</td>
<td>36.77</td>
</tr>
<tr>
<td>1000</td>
<td>91.3 ± 1.5</td>
<td>44.6 ± 1.4**</td>
<td>51.15</td>
</tr>
<tr>
<td>1500</td>
<td>84.8 ± 1.6</td>
<td>32.5 ± 1.8**</td>
<td>61.68</td>
</tr>
<tr>
<td>0.1 (Atro.)</td>
<td>86.2 ± 2.0</td>
<td>30.5 ± 1.8**</td>
<td>64.6</td>
</tr>
<tr>
<td>1.0 (yoh.)</td>
<td>88.7 ± 5.3</td>
<td>53.1 ± 1.1**</td>
<td>40.17</td>
</tr>
<tr>
<td>+1500</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.01;
** P < 0.001, significance relative to control.
*% increase; yoh., yohimbine; Atro., atropine; n = 5.

3.3. Intestinal fluid accumulation

There was a dose dependent decrease in intestinal fluid accumulation. The extract 500–1500 mg/kg reduced the intestinal fluid accumulation from 25.44 to 37.87% relative to control (Table 3).

3.4. Indomethacin-induced gastric ulceration

The extract (p.o) pretreatment on indomethacin-induced gastric ulceration showed a progressive decline in ulcer indices in pretreated groups relative to control (P < 0.05–0.001). The preventive ratio (percentage protection) also showed an ascending pattern (Table 4).

3.5. Ethanol-induced gastric ulceration

The extract significantly protected rats from ethanol-induced ulcers (Table 5). There was significant (P < 0.01–0.001) reduction in the ulcer indices relative to control. Propranolol, a β-adrenergic blocker, reduced the ulcer index caused by ethanol by 21.74%.

4. Discussion

Methanolic extract of *A. pubescens* root inhibited dose-dependently the small intestinal propulsion movement (IPM) in rat. The data suggests that this effect on IPM is mediated by α2-adrenoceptor stimulation because α2-adrenoceptor antagonists, yohimbine, significantly reduced the extract-induced transit delay in rats. The result further supports the idea that activation of α2-adrenoceptor induce delay in IPM (Ruwarts et al., 1980; Hsu, 1982).

The extract also showed a dose-related decrease in castor oil-induced diarrhoea. Drugs affecting motility, frequency and consistency of diarrhoea

Table 2
Effect of *Asparagus pubescens* extract on castor oil-induced diarrhoea in rats*

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Total no. of faceal matter</th>
<th>% Reduction (inhibition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml saline</td>
<td>79</td>
<td>–</td>
</tr>
<tr>
<td>500</td>
<td>56</td>
<td>29.11</td>
</tr>
<tr>
<td>1500</td>
<td>48</td>
<td>39.24</td>
</tr>
<tr>
<td>5.0 (diph)+1500</td>
<td>13</td>
<td>83.54</td>
</tr>
<tr>
<td>1.0 (yoh.)+1500</td>
<td>56</td>
<td>29.11</td>
</tr>
</tbody>
</table>

* diph, diphenoxylate; yoh., yohimbine; n = 5.

Table 3
Effects of *Asparagus pubescens* extract on castor oil-induced intestinal fluid accumulation in rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Vol. of intestinal fluid (ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ml saline</td>
<td>0.98 ± 0.07</td>
<td>–</td>
</tr>
<tr>
<td>2 ml castor oil</td>
<td>3.38 ± 0.33</td>
<td>–</td>
</tr>
<tr>
<td>500</td>
<td>2.52 ± 0.62*</td>
<td>25.44</td>
</tr>
<tr>
<td>1500</td>
<td>2.10 ± 0.08**</td>
<td>37.87</td>
</tr>
</tbody>
</table>

* P < 0.3;
** P < 0.01, significance relative to control; n = 5.
Table 4
Effect of *Asparagus pubescens* extract on indomethacin-induced ulceration in rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Ulcer index</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.90 ± 4.05</td>
<td>–</td>
</tr>
<tr>
<td>500</td>
<td>10.70 ± 1.40*</td>
<td>51.14</td>
</tr>
<tr>
<td>1000</td>
<td>3.80 ± 1.76**</td>
<td>82.65</td>
</tr>
<tr>
<td>1500</td>
<td>1.04 ± 0.28**</td>
<td>95.25</td>
</tr>
</tbody>
</table>

* P < 0.05; ** P < 0.001, significance relative to control; n = 5.

Table 5
Effect of *Asparagus pubescens* extract on ethanol-induced ulceration in rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Ulcer index</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.60 ± 0.29</td>
<td>–</td>
</tr>
<tr>
<td>250</td>
<td>3.0 ± 0.61*</td>
<td>34.78</td>
</tr>
<tr>
<td>500</td>
<td>2.4 ± 0.45**</td>
<td>47.83</td>
</tr>
<tr>
<td>1000</td>
<td>1.4 ± 0.45***</td>
<td>69.57</td>
</tr>
<tr>
<td>1500</td>
<td>0.80 ± 0.35***</td>
<td>82.60</td>
</tr>
<tr>
<td>40.0 (propr.)</td>
<td>3.60 ± 0.27*</td>
<td>21.74</td>
</tr>
</tbody>
</table>

* P < 0.01; ** P < 0.02; *** P < 0.001, significance relative to control; propr., propranolol; n = 5.

also affect secretion (DiCarlo et al., 1994). The intraluminal fluid accumulation induced by castor oil was blocked by the extract in the dose-related fashion. The involvement of α-adrenoceptor effect was further confirmed by the antagonistic action of yohimbine and α-adrenoceptor antagonist. All the result therefore suggest that the extract produced an inhibitory action on gastrointestinal functions, motility and secretion, and this effect is mediated through the activation of α<sub>2</sub>-adrenoceptor system.

The extract reduced both indomethacin and ethanol-induced ulceration in rats in dose-dependent manner. The mechanisms by which the extract produce these effects seem unclear.

Indomethacin is an established ulcerogen especially in an empty stomach (Bhargava et al., 1973). The incidence of indomethacin-induced ulceration is mostly on the glandular (mucosal) part of stomach (Elegbe and Bamgbose, 1976; Evbuonwa and Bolarinwa, 1990; Nwafor et al., 1996). Although the mechanisms underlying the ulcerogenicity of indomethacin are not completely understood, it has been known that inhibition of prostaglandin synthesis may be important (Vane, 1971). The view is supported by the fact that prostaglandins normally serve as protective function in stomach by maintaining gastric microcirculation (Vane, 1971; Ferreira and Vane, 1974) and causes gastric secretion of bicarbonate (Ganner et al., 1979) and mucus (Menguy and Desbaillets, 1967).

The incidence of ethanol-induced ulcers which is predominant in the glandular part of stomach has been reported to stimulate the formation of leukotriene C<sub>4</sub> (LTC<sub>4</sub>) resulting in the damage of rat gastric mucosa (Dreyling et al., 1986; Peskar et al., 1986; Cho et al., 1987). Indeed, in the rat gastric mucosa, some of the effects elicited by exogenous LTC<sub>4</sub> resemble those produced by ethanol (Guth et al., 1984; Szabo et al., 1985; Whittle et al., 1985).

The extract inhibited both the indomethacin and ethanol-induced ulceration in rat. It has been proposed that mucosal protection induced by nonprostanoid compounds may be mediated through the mobilization of endogenous prostaglandins (Cho et al., 1983; Konturek et al., 1987). It is possible that one of the mechanisms of antiulcerogenic effects of the extract may be due to its ability to mobilize prostaglandins in gastric mucosa by increasing its microcirculation or through an unknown mechanism.

The phytochemical analysis of the extract revealed the presence of tannins, saponins and flavonoids (Nwafor et al., 1998), substances known to affect the integrity of mucous membranes (Oliver, 1960). Tannins with its protein precipitating and vasoconstrictory effects could be advantageous in preventing ulcer development (Agwu and Nwako, 1988). Tannins being an astrigent, may have precipitated microproteins on the site of the ulcer thereby forming an impervious protective pellicle over the lining to prevent absorption of toxic substances and resist the attack of proteolytic enzymes (John and Onabanjo, 1990; Nwafor et al., 1996). Flavonoids have been reported to offer some protection in ulcer development by increasing capillary resistance.
Flavonoids improve microcirculation which renders the cells less injurious to precipitating factors (Hashizume et al., 1978).

Acknowledgements

The authors gratefully acknowledge the Department of Pharmacology and Clinical Pharmacy, University of Jos for providing laboratory space and Mr Peter Nggada for typing the manuscript. This work was supported by University of Maiduguri Fellowship Research Grant, and University of Jos Senate Research grant (No. RGC/1994/95/10) awarded to FKO.

References


Garlic enhances circulatory antioxidants during 7,12-dimethylbenz[a]anthracene–induced hamster buccal pouch carcinogenesis

S. Balasenthil, S. Arivazhagan, S. Nagini *

Department of Biochemistry, Faculty of Science, Annamalai University, Annamalainagar 608 002, Tamil Nadu, India

Received 24 March 1999; received in revised form 9 November 1999; accepted 28 April 2000

Abstract

The protective effect of garlic (Allium sativum Linn.) on circulatory lipid peroxidation and antioxidants was investigated during 7, 12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis in male Syrian hamsters. Enhanced lipid peroxidation in the circulation of tumour-bearing animals was accompanied by a significant decrease in the levels of ascorbic acid, vitamin E, reduced glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase. Administration of garlic extract significantly decreased lipid peroxidation with simultaneous depletion of antioxidants. We speculate that garlic exerts its protective effects by decreasing circulatory lipid peroxides and enhancing antioxidants. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Oral cancer; Garlic; DMBA; Lipid peroxidation; Antioxidants; Chemoprevention

1. Introduction

Garlic (Allium sativum Linn.), used as a spice and medicinal herb, exhibits a wide range of properties including immunomodulatory, hepatoprotective, antioxidant, antimutagenic and anticarcinogenic effects (Horie et al., 1989; Agarwal, 1996). The anticarcinogenic property of garlic has been documented from both epidemiological and experimental studies (Gao et al., 1999; Siegers et al., 1999). In previous studies from this laboratory, we have shown a positive correlation between the chemopreventive potential of garlic and its inducing effects on antioxidants and detoxification systems during DMBA-induced hamster buccal pouch carcinogenesis and N-methyl N’-nitro-N-nitrosoguanidine induced gastric carcinogenesis (Arivazhagan et al., 2000, 1999a,b, 2000; Balasenthil and Nagini, 1998; Balasenthil et al., 1999).

Assay of circulatory biomarkers has emerged as a reliable method for screening putative chemopreventive agents. Circulatory levels of lipid peroxidation and antioxidants are reliable indicators,
because they reflect the bioavailability as well as increased utilisation to counter lipid peroxidation. Previously, we demonstrated the importance of circulatory biomarkers to monitor the chemopreventive potential of plant products against 4-nitroquinoline1-oxide-induced oral carcinogenesis (Nagini and Manoharan, 1997).

The present study was undertaken to assess the chemopreventive potential of garlic against DMBA-induced hamster buccal pouch carcinogenesis using circulatory lipid peroxidation and the antioxidants GSH, ascorbic acid, vitamin E, GPx, SOD and catalase as biomarkers.

2. Materials and methods

2.1. Preparation of garlic extract

Garlic was purchased from the local market. The plant was identified and authenticated at the Herbarium of Botany Directorate in Annamalai University. A voucher specimen (104 J) was deposited in the Department of Botany, Annamalai University.

The dose of garlic was calculated on the basis of the weight of fresh garlic in mg used to prepare 1 ml extract. An aqueous extract of fresh garlic was prepared by homogenizing required amount of freshly peeled cloves in an appropriate volume of double distilled water to give a concentration of 25 mg ml\(^{-1}\) (Singh et al. 1996). The homogenate was centrifuged at 3120 \(g\) for 10 min to remove the particulate matter and the supernatant fraction was used for the experiment. At this stage of preparation, 96% of the extract was remaining.

2.2. Chemicals

DMBA was purchased from Sigma Chemical Company (St Louis, MO, USA). All other reagents used were of analytical grade.

2.3. Animals

All the experiments were carried out with male Syrian hamsters aged 8–10 weeks obtained from the Central Animal House, Annamalai University, India. They were housed six in a polypropylene cage and provided food and water ad libitum. The animals were maintained in a controlled environment under standard conditions of temperature and humidity with an alternating 12 h light/dark cycles. All animals were fed standard pellet diet (Mysore Snack Feed Ltd., Mysore, India).

2.4. Treatment Schedule

The animals were randomized into experimental and control groups and divided into four groups of six animals each. Animals in group 1 were painted with a 0.5% solution of DMBA in liquid paraffin on the right buccal pouches using a number four brush three times per week for 14 weeks. Each application leaves approximately 0.4 mg DMBA (Shklar, 1972). Group 2 animals were painted with DMBA as in group 1. In addition, the animals were administered 250 mg kg\(^{-1}\) body weight garlic extract orally three times per week on days alternate to DMBA application (Singh et al., 1996). Animals in group 3 received only garlic extract as in group 2. Group 4 (untreated control) animals received neither DMBA nor garlic extract. The experiment was terminated at the end of 14 weeks and all animals were killed by cervical dislocation after an overnight fast.

Biochemical parameters were carried out in blood samples of experimental and control animals. Blood samples were collected in heparinized tubes and plasma was separated by centrifugation at 1000 \(g\) for 15 min.

2.5. Preparation of hemolysate

After centrifugation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of erythrocytes was lysed with hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at 2500 \(g\) for 15 min at 2°C.

2.6. Estimations

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances...
(TBARS) was assayed in plasma by the method of Yagi (1978) and in erythrocytes by the method of Donnan (1950). Ascorbic acid, vitamin E and glutathione were estimated by the methods of Omaye et al. (1979), Baker et al. (1980) and Ellman (1959), respectively. GPx activity was assayed by following the utilisation of hydrogen peroxide according to the method of Rotruck et al. (1973). Superoxide dismutase was assayed by the method of Kakkar et al. (1984) and catalase by the method of Sinha (1972). Haemoglobin in erythrocytes and haemolysate was measured according to the method of Drabkin and Austin (1932).

2.7. Statistical analysis

Statistical analysis for the incidence of lesions was performed using Fisher’s exact probability test. The data for TBARS and antioxidants were analysed using analysis of variance (ANOVA) and the group means were compared by the Duncan’s multiple range test (DMRT). The results were considered statistically significant if the $P$ value was 0.05 or less.

3. Results

Table 1 summarises the incidence of oral neoplasms and preneoplastic lesions in different groups. Exophytic tumours induced by DMBA in the oral cavity of hamsters in group 1 were well differentiated squamous cell carcinomas. The incidence of oral neoplasms in group 1 was 100%, whereas in group 2, only early preneoplastic lesions such as hyperplasia were observed. No malignant neoplasms or premalignant lesions were seen in animals of groups 3 and 4.

The levels of lipid peroxidation in plasma and erythrocytes of control and experimental animals in each group are shown in Table 2. Lipid perox-

Table 1
Incidence of tumours and preneoplastic lesions

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of hamsters examined</th>
<th>Simple hyperplasia</th>
<th>Dysplasia</th>
<th>Squamous cell carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMBA (0.5%)</td>
<td>6</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>2</td>
<td>DMBA + Garlic (250 mg kg$^{-1}$ bw)</td>
<td>6</td>
<td>3 (50)*</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>Garlic (250 mg kg$^{-1}$ bw)</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Significantly different from group 1 by Fisher’s exact probability test. Values are statistically significant at $P<0.05$. Parentheses, percentage of lesions.

Table 2
TBARS in plasma and erythrocytes of control and experimental animals*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Plasma TBARS (nmoles ml$^{-1}$)</th>
<th>Erythrocyte TBARS (Pnoles/mg Hb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMBA (0.5%)</td>
<td>4.22 ± 0.35$^A$</td>
<td>2.82 ± 0.11$^A$</td>
</tr>
<tr>
<td>2</td>
<td>DMBA + Garlic (250 mg kg$^{-1}$ bw)</td>
<td>3.32 ± 0.46$^B$</td>
<td>1.81 ± 0.15$^B$</td>
</tr>
<tr>
<td>3</td>
<td>Garlic (250 mg kg$^{-1}$ bw)</td>
<td>2.19 ± 0.27$^D$</td>
<td>1.31 ± 0.13$^D$</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>2.85 ± 0.26$^C$</td>
<td>1.55 ± 0.14$^C$</td>
</tr>
</tbody>
</table>

* Values not sharing a common superscript letter differ significantly at $P<0.05$; (mean ± SD; $n = 6$)
Table 3
Antioxidants in plasma and erythrocytes and erythrocyte lysate of control and experimental animals*

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>1 DMBA</th>
<th>2 DMBA + Garlic</th>
<th>3 Garlic</th>
<th>4 Control</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GSH (mg dl⁻¹)</td>
<td>24.93 ± 3.57D</td>
<td>29.85 ± 3.30C</td>
<td>42.60 ± 4.31A</td>
<td>34.91 ± 3.51B</td>
<td></td>
</tr>
<tr>
<td>Vitamin C (mg dl⁻¹)</td>
<td>0.65 ± 0.05D</td>
<td>0.98 ± 0.07C</td>
<td>1.45 ± 0.08A</td>
<td>1.20 ± 0.03B</td>
<td></td>
</tr>
<tr>
<td>Vitamin E (mg dl⁻¹)</td>
<td>0.52 ± 0.03D</td>
<td>0.78 ± 0.04C</td>
<td>1.38 ± 0.05A</td>
<td>1.11 ± 0.08B</td>
<td></td>
</tr>
</tbody>
</table>

| **Erythrocytes** | | | | | |
| GSH (mg dl⁻¹) | 23.68 ± 3.79D | 32.78 ± 2.89C | 53.23 ± 4.28A | 42.33 ± 3.48B |

| **Erythrocyte lysate** | | | | | |
| GPx (U g⁻¹ Hb) | 12.10 ± 2.90D | 18.47 ± 2.41C | 32.23 ± 3.79A | 22.78 ± 3.17B |
| SOD (U mg⁻¹ Hb) | 1.87 ± 0.14D | 2.42 ± 0.12C | 3.67 ± 0.11A | 2.92 ± 0.18B |
| Catalase (U mg⁻¹ Hb) | 1.10 ± 0.09D | 1.44 ± 0.10C | 2.26 ± 0.13A | 1.62 ± 0.11B |

* Values not sharing a common superscript letter differ significantly at P<0.05; (mean ± SD; n = 6).

a μmoles of glutathione utilised min⁻¹.

b The amount of enzyme required to inhibit 50% NBT reduction.

c μmoles of H₂O₂ utilised min⁻¹.

idation was significantly increased in group 1 compared with group 4. In groups 2 and 3, the values were significantly lower compared with group 1.

Table 3 shows the levels of antioxidants in plasma, erythrocytes and erythrocyte lysate of experimental and control animals in each group. The levels of ascorbic acid, vitamin E, glutathione, GPx, SOD and catalase were significantly lower in group 1 compared with group 4. In groups 2 and 3, the levels of ascorbic acid, vitamin E, glutathione, as well as the activities of GPx, SOD and catalase were significantly higher compared to group 1.

4. Discussion

Enhanced lipid peroxidation associated with antioxidant depletion in circulation is a characteristic finding in malignant transformation. Free radicals, which are highly toxic, traverse membranes and cause deleterious effects at sites far from the tumour (Dreher and Junod, 1996). The enhanced lipid peroxidation in circulation in hamsters bearing DMBA-induced oral tumours reflects excessive free radical generation exacerbated by a decreased efficiency of host antioxidant defence mechanisms.

The increase in lipid peroxidation was associated with a decrease in the antioxidants. Ascorbic acid is an essential antioxidant that disappears faster than other antioxidants when plasma is exposed to reactive oxygen species (Frei et al., 1989). Vitamin E is the major lipid soluble antioxidant present in plasma and erythrocyte membranes (Gerster, 1995). Glutathione, an important cellular reductant, offers protection against free radicals, peroxides and toxic compounds (Vina et al., 1989). The deficiency of ascorbic acid, vitamin E and glutathione in the circulation of tumour-bearing hamsters may be due to their increased utilisation to scavenge the products of lipid peroxidation. A decrease in the activities of GPx, SOD and catalase, the major cellular detoxifying enzyme systems, has been reported in malignancies (Corrocher et al., 1986; Arivazhagan et al., 1997). Our results are in line with these findings.

Administration of garlic extract reversed the changes induced by DMBA supporting the hypothesis that plant products are effective chemopreventive agents. Garlic has been reported to modulate lipid peroxidation levels and enhance the status of antioxidants (Arivazhagan et al., 2000, 1999a,b; Balasenthil and Nagini 1998; Balasenthil et al., 1999). Allium components have been reported to elevate the levels of GSH, GPx, SOD and catalase (Pinto et al., 1997; Wei and Lau, 1998). The beneficial effects of garlic can be attributed to the
presence of antioxidants such as vitamin C and other phytochemicals such as organosulphur compounds.

We speculate that garlic mediates its chemopreventive effects by decreasing lipid peroxidation and enhancing antioxidant status. The results of the present study together with our previous findings indicate that garlic may emerge as a putative chemopreventive agent against oral carcinogenesis.

References


Anxiolytic effect of seed of *Ziziphus jujuba* in mouse models of anxiety

Wen-Huang Peng a,*, Ming-Tsuen Hsieh a, Yi-Shung Lee a, Yi-Chin Lin a, Jen Liao b

a Institute of Chinese Pharmaceutical Sciences, China Medical College, Taichung, Taiwan, ROC
b Taichung Municipal Jen-Ai Hospital, Taichung, Taiwan, ROC

Received 30 November 1999; received in revised form 22 March 2000; accepted 28 April 2000

Abstract

The aim of the present study was to investigate the anxiolytic effect of *Semen Ziziphi jujuba* (SZJE). The SZJE was administered orally to male ICR mice, at 0.5, 1.0 and 2.0 g/kg, 30 min before the behavioral evaluation in the black and white test (BWT) and elevated plus maze (EPM). The SZJE at the dosage 0.5–2.0 g/kg increased the first time entry, total changes and times spent in the white chamber of the BWT. The SZJE at the dosage 0.5–1.0 g/kg increased the percentage of time-spent and the percentage of arm entries in the open arms of the EPM and decreased the percentage of time-spent and the percentage of arm entries in the closed arms of the EPM. Furthermore, the SZJE at the dosage of 1.0 g/kg prolonged the hexobarbital-induced sleeping time in mice and decreased the locomotor activity in rats. These results suggested that SZJE possessed anxiolytic effect at lower dose and sedative effect at higher dose. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Semen Ziziphi jujuba*; Anxiolytic effect; Black and white test; Elevated plus maze

1. Introduction

In traditional medicine *Semen Ziziphi Jujuba* (SZJ), the seed of *Z. jujuba* Mill. (Rhamnaceae) has been used for its action on insomnia and anxiety (Lee, 1986). In the modern pharmacological studies, SZJ possesses the hypnotic-sedative, hypotensive, antihypoxia, antihyperlipidemia, and hypothermic effects (Yen, 1991). Suanzaozentang, a prescription of the Chinese Medicine, possesses the anxiolytic effect in the clinical test. Its action of mechanism was as a result of decreasing the monoaminergic system activity (Hsieh et al., 1986). SZJ is the major component of Suanzaozentang. However, the anxiolytic effect of the SZJ was never been studied.

Anxiety has became a very important area of research interest in psychopharmacology this decade. This increased interest is as a result of a rapid growth of scientific studies and the discovery of new drugs that alter anxiety in animal models. Furthermore, anxiety disorders are appreciated to be very prevalent in community surveys.
(Regier et al., 1988) and the economic cost of them well justifies this increase in research interest and the development of new pharmacological approaches. Black and White test (Crawley and Goodwin, 1980; Crawley, 1981), which uses the aversion of rodents to brightly lit large spaces and the elevated plus-maze, is a widely used test based on the natural aversion of rodents to heights and open spaces, which has been validated for both mice and rats (Lister, 1987; Dawson and Tricklebank, 1995; Helton et al., 1996) and originally developed by Pellow et al. (1985), are two simple yet sensitive tests that assess anxiety in rodents.

Clinically proven anxiolytics such as diazepam (Valium) and buspirone (Buspar) are effective in the two models (Helton et al., 1996). However, all clinically available anxiolytics have limited clinical efficacy because of their adverse side effect of sedation. Therefore, the aims of this study were attempted to investigate the anxiolytic effects of the ethanolic extract of SZJ in the two animal models, the black white test and the elevated plus-maze. We also profiled the secondary pharmacology of the SZJE on spontaneous activity and interaction with a CNS depressant.

2. Materials and methods

2.1. Preparation of the plant material

SZJ was purchased from Sheng-Long Chinese herbal store, Taichung, Taiwan and was authenticated by Dr Chung-Chuen Chen, Institute of Chinese Pharmaceutical Sciences, China Medical College, Taichung, Taiwan. A voucher specimen (No. 182) was deposited in the Institute of Chinese Pharmaceutical Sciences of China Medical College. The seed was dried and placed in round-bottom glass bottles. Three liters of 95% ethanol were added to each and reflux for 4 h. The procedure was repeated twice. The extracts were filtered and then concentrated to dryness in a rotavapor in vacuum at 50°C. We obtained the ethanolic extract of SZJ (abbreviation SZJE). The yield of the extract was 23.34% (w/w).

2.2. Animals

All experiments were performed using male ICR mice (18–25 g) which were obtained from the Animal Centre of the China Medical College, Taichung, Taiwan. They were housed in groups of six in a room with a 12 L:12 D cycle and maintained on laboratory pellets (Fu-So Inc., Taichung) and tapwater ad lib. Animals were naive to an BWT and EPM.

2.3. Drugs

Diazepam was obtained from Siu-Guan Chemical Works (Taipei City, ROC) in a solvent form (10 mg/2 ml ampoule). It was diluted with 0.9% saline to 1 mg/kg before use. Buspirone hydrochloride was purchased from Sigma (St. Louis, MO, USA) and was dissolved in 0.9% saline.

2.4. Black and white test

The apparatus consisted of two fully automated polyvinylchloride boxes monitored by computer. Boxes, divided into a small chamber (27 x 18 x 45 cm) and a large chamber (27 x 27 x 45 cm), with an opening door (7 x 7 cm) located in the center of the partition at floor level. The small chamber was painted black and illuminated under a 60 W red light. The large chamber was painted white and brightly illuminated with a 60 W light source. Bulbs were located 37 cm above the floor. The chambers were equipped with infrared beam sensors (four in the white area, three in the black one), enabling the detection of time spent in each zone, latency of the first crossing from white chamber to black chamber and shuttle crossings between two chambers. The data from these three parameters were directly collected by computer.

The test was performed in a quiet, darkened room. The mice was kept in this room at least 1 h before the test. After administration (saline or treatment), mice were placed in their home cage. Naive mice are placed individually in the middle of the light area facing away from the opening. A 5-min test is given, during which the three parameters are recorded. All experiments were carried out between 10:00 and 14:00 h. Buspirone...
(2 mg/kg, i.p.) and diazepam (1.0 mg/kg, i.p.) were used as the positive control. The animals were introduced into the apparatus 30 min after SZJE administration.

2.5. Elevated plus-maze

The elevated plus-maze was a modification of the apparatus validated for mice by Lister (Lister, 1987) and consisted of two open arms (30 × 5 × 0.25 cm) and two closed arms (30 × 5 × 15 cm) emanating from a common central platform (5 × 5 cm). The two pairs of identical arms were opposite each other. The entire apparatus was elevated to a height of 50 cm above floor level. At the start of the session, the mouse was placed at the center of the maze, its head facing a open arm and allowed to explore the maze for 5 min. A video camera mounted overhead, connected to a videomex-one (Columbus, USA), recorded the behavior of the mouse on the maze for subsequent analysis. The following parameters were scored: the percentage of time spent and percentage of arm entries in each type of arms.

The plus maze was carefully wiped with a wet towel after each animal. Buspirone (2 mg/kg, i.p.) and Diazepam (1.0 mg/kg, i.p.) were used as the positive control. All experiments were carried out between 10:00 and 14:00 h.

2.6. Hexobarbital-induced sleeping time

Mice were given a single oral dose of SZJE (0.5–1.0 g/kg) 30 min before challenge with hexobarbital (100 mg/kg, i.p.). Observation of the mice
began immediately after hexobarbital injection. Once the mouse was asleep, it was transferred to a prenumbered chamber and placed on its back. Mice was considered awake when they could successfully right themselves (all four feet in contact with the surface). Once a mouse righted itself, it was placed its back once more and allowed to right a second time for conformation. Sleep duration was recorded for each mouse.

2.7. Ambulatory behavior test

Immediately following SZJE administrations, each rat was placed in an Omnitech Digiscan Animal Activity Monitor (Model Opto-Varimex, Columbus Co., USA) for 60 min. The acrylic cage within the monitor measured approximately 42 wide by 42 long and 31 cm high. The monitor was equipped with 16 beams 2.54 cm apart from front
to back and from side to side, as well as 16 beams 2.54 cm apart from side to side on the upper level. Every 100 ms, the computer sampled the status of all the beams. The Digiscan analyzer converted the patterns of beams broken into different measures of locomotor activity. In this study, the measure automatically analyzed by the computer is the horizontal movement counts.

2.8. Statistics

All data obtained from the BWT are expressed in term of medians and interquartile ranges. Statistical analysis was performed using the non-parametric Kruskal–Wallis, \( H \)-test, followed by Mann–Whitney’s \( U \)-test. In addition, the data of EPM and ambulatory behavior test are expressed as mean ± S.E.M. and analyzed using one-way ANOVA followed by Scheffé’s test. \( P < 0.05 \) was considered significant.

3. Results

3.1. Effects of SZJE on the BWT

SZJE at 0.5, 1.0, 2.0 g/kg increased the first time entry \( (P < 0.001; \text{Fig. } 1) \), time spent in the white chamber \( (P < 0.01–0.001; \text{Fig. } 2) \), total changes between two chambers \( (P < 0.05–0.001; \text{Fig. } 3) \), and decreased the time spent in the black chamber \( (P < 0.01–0.001; \text{Fig. } 2) \).

3.2. Effect of SZJE on the EPM

As shown in Fig. 4, SZJE at 0.5 and 1.0 g/kg increased the percentage of arm entries and percentage of time spent in the open arms, decreased the percentage of arm entries and percentage of time spent in the closed arms.

3.3. Effects of SZJE on the hexobarbital-induced hypnosis in mice

As shown in Fig. 5, SZJE at 1.0 g/kg prolonged the hexobarbital-induced sleeping time.

3.4. Effects of SZJE on the locomotor activity in rats

As shown in Fig. 6, SZJE at 1.0 g/kg decreased the locomotor activity in rats.

4. Discussion

In the present study, we used animal models of anxiety such as the BWT and the EPM to mea-
sure the anxiolytic effect of SZJE. The behaviour observed using the BWT in the present study confirmed the anxiolytic activity of diazepam and buspirone as reported previously (Corda and Biggio, 1986; Carli et al., 1989). SZJE (0.5, 1.0 and 2.0 g/kg) increased the time spent in the white chamber, first time entry from white chamber to the black chamber and total changes between the two chambers. The results indicated that SZJE possessed the anxiolytic effect in the BWT in rodents.

In the elevated plus-maze, the behaviour observed using the EPM in the present study confirmed the anxiolytic activity of buspirone and diazepam as reported previously (Soderpalm et al., 1989). Using this test, SZJE increased the percentage of time spent and percentage of arm entries in the open arms and decreased the percentage of time spent and percentage of arm entries in the closed arms. This test is based on a premise in which the exposure to an EPM evoked an approach-avoidance conflict that was considerably stronger than that evoked by exposure to an enclosed arms (Montgomery, 1955). The decreased aversion to the open arms is as a result of an anxiolytic effect expressed by an increased number of open arm entries and time-spent, and decreased the number of closed arm entries and time spent in the EPM. Therefore, the behavioural alterations induced by SZJE in the EPM are consistent with an anxiolytic effect.

Suanzaozentang, a prescription of traditional Chinese medicine, possesses anxiolytic effect. Its action of mechanism is related to the decrease in the monoaminergic activities (Hsieh et al., 1986). SZJ, the major component of Suanzaozentang, also could decreased the monoamine levels in the brain (Chang and Chen, 1995). The hypothesis that excess monoamines increases anxiety, while antagonism or reduction in monoamines activity is anxiolytic (Lane et al., 1982; Koprowska et al., 1999). Therefore, the anxiolytic mechanism of SZJE may be related to the decrease in the monoaminergic activity in the brain.

It is well known that many drugs such as benzodiazepines and phenobarbital possess anxiolytic and sedative effects (Treit, 1985). SZJ possesses sedative effect (Hong and Cao, 1987). In our present study, SZJE increased the hexobarbital-sleeping time and decreased the locomotor activity at the higher dose. These results shown that SZJE possessed anxiolytic effect at lower dose and sedative effect at higher dose. The major sedative components of SZJ are the spinosin and jujubosides (Yuan et al., 1987). It must be made further studies to clarify whether the major anxiolytic component of SZJE is spinosin and jujubosides or not.

In summary, the SZJE exhibits anxiolytic effect at lower dose. The major active components and precise anxiolytic mechanisms need to be identified, but they indicate the value of further inves-

![Fig. 6. Effects of diazepam (DIZ), buspirone (BUS) and the ethanol extract of SZJ (ESZJ) on the locomotor activity in rats. ***P < 0.001 as compared with the control group (one-way ANOVA following by Scheffe test).]
tigation of SZJE with known and possible future clinical application.

Acknowledgements

We are grateful to the Executive Yuan-National Health Administration for the financial support of this manuscript under contract CCMP86-RD-030 and CMC-85008.

References


Effect of *Vitex rotundifolia* on immediate-type allergic reaction

Tae-Yong Shin a, Sug-Hyun Kim a, Jong-Phil Lim a, Eun-Sil Suh a, Hyun-Ja Jeong b, Byung-Do Kim b, Eun- Jeung Park c, Woo-Jun Hwang c, Do-Gon Rye c, Seung-Hwa Baek d, Nyeon-Hyoung An b, Hyung-Min Kim b,*

a College of Pharmacy, Woosuk University, Wanju, Chonbuk, 565-701, South Korea
b College of Pharmacy and Center of Oriental Medicinal Science, Wonkwang University, Iksan, Chonbuk, 570-749, South Korea
c College of Oriental Medicine, Wonkwang University, Iksan, Chonbuk, 570-749, South Korea
d Department of Natural Products, Professional Graduate School of Oriental Medicine (BK 21), Wonkwang University, Iksan, Chonbuk, 570-749, South Korea

Received 11 October 2000; received in revised form 17 April 2000; accepted 1 May 2000

Abstract

We investigated the effect of aqueous extract of *Vitex rotundifolia* (L.) (Verbenaceae) fruits (VRFE) on the immediate-type allergic reactions in vivo and in vitro. VRFE (10⁻⁴–1.0 g/kg) dose-dependently inhibited systemic allergic reaction induced by compound 48/80. When VRFE was employed in a systemic allergic reaction test, the plasma histamine levels were reduced in a dose-dependent manner. VRFE (5 × 10⁻⁴ and 1.0 g/kg) inhibited passive cutaneous anaphylaxis activated by anti-dinitrophenyl (DNP) IgE. VRFE (10⁻³–1.0 mg/ml) also dose-dependently inhibited the histamine release from the rat peritoneal mast cells (RPMC) by compound 48/80 or anti-DNP IgE. Moreover, VRFE (10⁻³ mg/ml) had a significant inhibitory effect on anti-DNP IgE-induced tumor necrosis factor-α production from RPMC. These results suggest that VRFE may be beneficial in the regulation of immediate-type allergic reaction. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Vitex rotundifolia* fruits; Immediate-type allergic reaction; Compound 48/80; Rat peritoneal mast cells; Passive cutaneous anaphylaxis; Tumor necrosis factor-α

1. Introduction

As part of our continuing search for biologically active antiallergic agents from medicinal sources, *Vitex rotundifolia* was analyzed. The fruits of *V. rotundifolia* (L.) (Verbenaceae), well known as ‘Man Hyung Ja’ in Korea, have been
used against headache in upper respiratory infection (Kimura et al., 1996) and they still occupy an important place in traditional Oriental medicine for treatment of various allergic diseases through various administration routes in Korea. Mast cells play a major role in immediate-type allergic reaction by releasing chemical mediators such as histamine, serotonin and arachidonic acid metabolites (Tizard, 1995). Among the preformed and newly synthesized inflammatory substances released on the degranulation of mast cells, histamine is the best characterized and most potent vasoactive mediator implicated in the acute phase of immediate-type allergic reaction (Petersen et al., 1996). Mast cell degranulation can also be elicited by a number of positively charged substances, collectively known as the basic secretagogues of mast cells (Lagunoff et al., 1983). Compound 48/80 is one of the most potent secretagogues of mast cells (Ennis et al., 1980). It is a mixture of polymers synthesized by condensing N-methyl-p-methoxyphenyl ethylamine with formaldehyde (Baltzly et al., 1949), and its hypotensive effect, resulting from histamine release, was shown by Paton (1951). Compared with the natural process, a high concentration of compound 48/80 induces almost a 90% release of histamine from mast cells. Thus, an appropriate amount of compound 48/80 has been used as a direct and convenient reagent to study the mechanism of anaphylactic allergic reaction (Allansmith et al., 1989). The secretory response of mast cells can also be induced by aggregation of their cell surface-specific receptors for IgE by the corresponding antigen (Segal et al., 1977; Metzger et al., 1986; Alber et al., 1991). The passive cutaneous anaphylactic (PCA) reaction has been used as an animal model of allergic reaction for many years. The anti-IgE antibody has been established to induce PCA reaction as a typical model for the immediate-type allergic reaction. The skin of rat or guinea pig is a useful site for studying PCA (Saito and Nomura, 1989). Although mast cells also store small amounts of cytokines in their granules (Gordon and Galli, 1990), these cells dramatically increase their production of tumor necrosis factor-α (TNF-α), IL-6, and other cytokines within 30 min after their surface FcεRI are cross-linked with specific antigen (Burd et al., 1989; Plaut et al., 1989; Wodnar-Filipowicz et al., 1989; Gurish et al., 1991).

In the present study, we show that the aqueous extract of V. rotundifolia fruits (VRFE) inhibits both compound 48/80-induced systemic allergic reaction and anti-IgE antibody-induced cutaneous reaction. We also investigated the influence of VRFE on anti-dinitrophenyl (DNP) IgE-induced TNF-α production in rat peritoneal mast cells (RPMC).

2. Materials and methods

2.1. Materials

Compound 48/80, anti-(DNP) IgE, DNP-human serum albumin (HSA), bovine serum albumin (BSA), α-minimal essential medium (α-MEM), o-phthaldialdehyde (OPA) and metrizamide were purchased from Sigma (St Louis, MO). Fetal calf serum (FCS) was purchased from Life Technologies (Grand Island, NY). Murine TNF-α and anti-murine TNF-α were obtained from Genzyme (Cambridge, MA). Tissue culture flask and plates were obtained from Nunc (Naperville, IL).

2.2. Animals

The original stock of male Wistar rats weighing 200–300 g were purchased from Dae-Han Experimental Animal Center (Taejeon, Chungnam, South Korea), and the animals were kept at the College of Pharmacy, Woosuk University. The animals were housed 5–10 per cage in a laminar air flow room maintained at 22 ± 1°C and relative humidity of 55 ± 10% throughout the study.

2.3. Preparation of VRFE

The fruits of V. rotundifolia were purchased from the Oriental drug store, Bohwa Dang (Chunju, South Korea). A voucher specimen (Number W-1-29) was deposited at the Herbarium of the College of Pharmacy, Woosuk University. The fruits were extracted with distilled water
(100 g/500 ml) at 70°C for 5 h. The extract was filtered through a 0.45-μm filter, lyophilized, and kept at 4°C. The yield of dried extract (black color) from starting crude materials was about 8.9% (w/w). The dried extract was dissolved in saline or Tyrode buffer A (10 mM Hepes, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% bovine serum albumin) before use.

2.4. Compound 48/80-induced systemic allergic reaction

Rats were given an i.p. injection of 8 mg/kg of the mast cell degranulator, compound 48/80. VRFE was dissolved in saline and administered by i.p. injection ranging from 10⁻⁴ to 1.0 g/kg 1 h before the injection of compound 48/80 (n = 10/group). In a time-dependent experiment VRFE (1 g/kg) was injected i.p. 1 h before, 5 and 10 min after compound 48/80 injection (n = 10/group). The rats were monitored for 1 h after induction of anaphylactic shock.

2.5. PCA reaction

An IgE-dependent skin reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the rat’s tail vein. The anti-DNP IgE and DNP-HSA were diluted in PBS. The rats were injected intradermally with 0.5 μg (50 μl) of anti-DNP IgE into each of four dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later each rat received an injection of 1 mg of DNP-HSA in PBS containing 4% Evans blue (1:4) via tail vein. VRFE (5 × 10⁻¹–1.0 g/kg) was orally administered 1 h before the challenge. Thirty minutes after the challenge, the rats were sacrificed and the dorsal skin was removed for measurement of pigment area. The amount of dye was then determined colorimetrically after extraction with 1 ml of 1.0 M KOH and 9 ml of a mixture of acetone and phosphoric acid (5:13) based on the method of Katayama et al. (1978). The absorbance intensity of the extraction was measured at 620 nm in a spectrophotometer (Shimadzu, UV-1201, Japan), and the amount of dye was calculated with the Evans blue measuring-line.

2.6. Preparation of plasma and histamine determination

The blood was centrifuged at 400 × g for 10 min. The plasma was withdrawn and histamine content was measured by the OPA spectrofluorometric procedure of Shore et al. (1959). The fluorescent intensity was measured at 438 nm (excitation at 353 nm) in a spectrofluorometer (Shimadzu, RF-5301 PC, Japan).

2.7. Preparation of RPMC

RPMC were isolated as previously described (Kim et al., 1998). In brief, rats were anesthetized by ether, and 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄) containing 0.1% gelatin (Sigma) was injected into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by a Pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150 × g for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from major components of rat peritoneal cells, i.e. macrophages and small lymphocytes, according to the method described by Yurt et al. (1977). In brief, peritoneal cells suspended in 1 ml Tyrode buffer B were layered on 2 ml of metrizamide (22.5% w/v) and centrifuged at room temperature for 15 min at 400 × g. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml Tyrode buffer A. Mast cell preparations were about 95% pure as assessed by Toluidine blue staining. More than 97% of cells were viable, as judged by Trypan blue uptake.

2.8. Inhibition of histamine release

Purified RPMC were resuspended in Tyrode buffer A for the treatment of compound 48/80.
RPMC suspensions (2 × 10^5 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80 (5 μg/ml). The cells were preincubated with the VRFE preparations, and then washed and incubated (10 min) with the compound 48/80. RPMC suspensions (2 × 10^5 cells/ml) were also sensitized with anti-DNP IgE (10 μg/ml) for 6 h. The cells were preincubated with the VRFE at 37°C for 1 min prior to the challenge with DNP-HSA (1 μg/ml). The cells were separated from the released histamine by centrifugation at 400 × g for 5 min at 4°C. Residual histamine in cells was released by disrupting the cells with perchloric acid and centrifugation at 400 × g for 5 min 4°C.

2.9. Assay of histamine release

The inhibition percentage of histamine release was calculated using the following equation:

\[
\text{% Inhibition} = \frac{A - B}{A} \times 100
\]

where \(A\) is histamine release without VRFE and \(B\), histamine release with VRFE.

### Table 1

<table>
<thead>
<tr>
<th>VRFE addition (g/kg)</th>
<th>Compound 48/80 (8 mg/kg)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (saline)</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>10^{-4}</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>10^{-3}</td>
<td>+</td>
<td>90</td>
</tr>
<tr>
<td>10^{-2}</td>
<td>+</td>
<td>80</td>
</tr>
<tr>
<td>10^{-1}</td>
<td>+</td>
<td>13 ± 7</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>

*Groups of rats were i.p. pretreated with 200 μl saline or VRFE was given at various doses 1 h before (\(n = 10\)/group) compound 48/80 injection.

*Compound 48/80 solution was i.p. given to the group of rats.

*Mortality (%) within 1 h following compound 48/80 injection is presented as no. of dead rats \times 100/total no. of experimental rats. Each datum represents the mean ± S.E. of three independent experiments.

2.10. Assay of TNF-α release

TNF-α secretion was measured by a modified enzyme linked immunosorbent assay (ELISA) as described (Kim and Lee, 1999). The ELISA was sensitive to TNF-α concentrations in the medium above 40 pg/ml. The ELISA was devised by coating 96-well plates with 6.25 ng/well of murine monoclonal antibody with the specificity for murine TNF-α. Before use and between subsequent steps in the assay, the coated plates were washed twice with PBS containing 0.05% Tween-20 and twice with PBS alone. All reagents used in this assay and the coated wells were incubated for 1 h at room temperature. For the standard curve, rTNF-α was added to plasma previously determined to be negative for endogenous TNF-α. After exposure to the medium, the assay plates were sequentially exposed to rabbit anti-TNF-α, phosphatase-conjugated goat anti-rabbit IgG, and 2,2’-azinobis.

2.11. Statistical analysis

Date are presented as means ± S.E. The Student’s \(t\)-test for unpaired observations was used to make a statistical comparison between the groups. Results with \(P < 0.05\) were considered statistically significant.

3. Results

3.1. Effect of VRFE on compound 48/80-induced systemic allergic reaction

To assess the contribution of VRFE in immediate-type allergic reactions, we first used the in vivo model of systemic allergic reaction. We used compound 48/80 (8 mg/kg) as a shock inducer. After the peritoneal injection of compound 48/80, the rats were monitored for 1 h, after which the mortality rate was determined. As shown in Table 1, i.p. injection of 200 μl saline as control induced
Table 2
Time-dependent effect of VRFE on compound 48/80-induced systemic allergic reaction

<table>
<thead>
<tr>
<th>VRFE addition (g/kg)a</th>
<th>Compound 48/80 (8 mg/kg)b</th>
<th>Mortality (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 h before</td>
</tr>
<tr>
<td>None (saline)</td>
<td>+</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>−</td>
<td>0</td>
</tr>
</tbody>
</table>

* Groups of rats were i.p. pretreated with 200 μl saline or VRFE was given at various times (n = 10/group) before or after compound 48/80 injection.
  
  b Compound 48/80 solution was i.p. given to the group of rats.
  
  c Mortality (%) within 1 h following compound 48/80 injection is presented as no. of dead rats × 100/total no. of experimental rats. Each datum represents the mean ± S.E. of three independent experiments.

fatal shock in 100% of each group. When rats were pretreated with VRFE at concentrations ranging from 10^-4 to 1.0 g/kg for 1 h, the mortality with compound 48/80 was reduced dose-dependently. Furthermore, the mortality of rats injected i.p. with VRFE (1.0 g/kg) 5 and 10 min after compound 48/80 injection was 0% (Table 2).

3.2. Effect of VRFE on plasma histamine release

The ability of VRFE to influence compound 48/80-induced plasma histamine release was investigated. VRFE was given from 10^-4 to 1.0 g/kg 1 h before (n = 5/group) compound 48/80 injection. The plasma of rats was uniformly gathered with heart puncher 15 min after compound 48/80 injection in all groups of rats. Similar results with those of the mortality test were shown when their plasma histamine contents were measured (Fig. 1).

3.3. Effect of VRFE on PCA reaction

The way to test local allergic reaction is to induce PCA (Wershil et al., 1987). As described in experimental procedures, local extravasation is induced by local injection of anti-DNP IgE followed by an i.v. antigenic challenge. Anti-DNP IgE was injected in the right dorsal skin sites. As a control, the left dorsal skin site of these rats was injected with saline alone. After 48 h, all animals were injected i.v. with DNP-HSA injected with Evans blue dye. The cutaneous allergic reaction

Fig. 1. Effect of VRFE on compound 48/80-induced plasma histamine release. Groups of rats were i.p. pretreated with 200 μl saline or VRFE. Each drug was given with various doses 1 h before compound 48/80 injection. Compound 48/80 solution (8 mg/kg) was i.p. given to the group of rats. Each datum represents the mean ± S.E. of three independent experiments. *P < 0.05; significantly different from the saline value.

Table 3
Effect of VRFE on 48 h PCA in rats

<table>
<thead>
<tr>
<th>VRFE addition (g/kg)a</th>
<th>Amount of dye (μg/site)b</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (saline)</td>
<td>9.3 ± 0.23</td>
<td>–</td>
</tr>
<tr>
<td>5 × 10^-1</td>
<td>5.6 ± 0.71</td>
<td>39.8*</td>
</tr>
<tr>
<td>1.0</td>
<td>4.1 ± 0.16</td>
<td>55.9*</td>
</tr>
</tbody>
</table>

* VRFE was administered orally 1 h prior to the challenge with antigen.
  
  b Each datum represents the mean ± S.E. of three independent experiments.
  
  * P < 0.05; significantly different from the saline value.
Finally, we investigated the ability of VRFE to influence anti-DNP IgE-induced TNF-α production in RPMC. VRFE inhibited TNF-α production at a concentration of $10^{-3}$ mg/ml (Table 4). No significant cytotoxicity of the VRFE on culture was observed in the concentrations used in the above experiments as assessed by Trypan blue uptake.

### 4. Discussion

The present study showed that VRFE treatment profoundly affected compound 48/80-induced systemic allergic reaction and anti-DNP IgE-induced PCA reaction. VRFE inhibited the release of histamine induced by the nonimmunologic compound as well as specific allergic mechanism from mast cells. We simply speculate that these results indicate that mast cell mediated immediate-type allergic reactions are inhibited by VRFE. There is no doubt that stimulation of mast cells with compound 48/80 initiates the activation of signal-transduction pathway which leads to histamine release. However, there remains a possibility that the VRFE contains lots of anionic polysaccharides, tannins and lots of compounds capable to bind and to inactivate minor amounts of 48/80, leading to diminished mast cell degranulation. To exclude this possibility, we incubated a VRFE solution with 1 g/ml plus 8 mg compound 48/80 in vitro for 1 h, and centrifuged down a potential precipitate and again tested the supernatant on remaining compound 48/80 activity. The test showed similar activity to the original compound 48/80. Moreover, we assayed compound 48/80-induced histamine release after washing the VRFE-treated RPMC. Some recent studies (Mousli et al., 1990a,b) have shown that compound 48/80 and other polybasic compounds are able, apparently directly, to activate G-proteins. The evidence indicates that the protein is Gi-like and that the activation is inhibited by benzalkonium chloride (Bueb et al., 1990).

Tasaka et al. (1986) reported that compound 48/80 increased the permeability of the lipid bi-

**Table 4**

<table>
<thead>
<tr>
<th>VRFE addition (mg/ml)</th>
<th>Anti-DNP IgE plus DNP-HSA</th>
<th>TNF-α production (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (saline)</td>
<td>–</td>
<td>1.232</td>
</tr>
<tr>
<td>None (saline)</td>
<td>+</td>
<td>1.847</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>+</td>
<td>1.600</td>
</tr>
</tbody>
</table>

*Effect of VRFE on anti-DNP IgE-induced TNF-α production.

RPMC (1.0 $\times$ 10⁶ cells/ml) were sensitized with anti-DNP IgE (1 μg/ml) for 16 h and incubated for 20 min VRFE before the challenge with DNP-HSA (0.1 μg/ml) for 4 h.

Each datum represents the mean of three independent experiments.

was best visualized by the extravasation of the dye. VRFE (5 $\times$ 10⁻¹ and 1.0 g/kg) inhibited PCA reaction significantly (Table 3).

### 3.4. Effect of VRFE on histamine release from RPMC

The inhibitory effect of VRFE on compound 48/80 or anti-DNP IgE-induced histamine release from RPMC are shown in Fig. 2. VRFE inhibited compound 48/80 or anti-DNP IgE-induced histamine release at concentrations of $10^{-3}$–1.0 mg/ml.

### 3.5. Effect of VRFE on TNF-α production

Fig. 2. Effect of VRFE on compound 48/80 (○)-induced or IgE-mediated (●) histamine release from RPMC. The cells (5 $\times$ 10⁵ cells/ml) were preincubated with VRFE at 37°C for 10 min prior to incubation with compound 48/80. The cells (2 $\times$ 10⁵ cells/ml) were preincubated with VRFE at 37°C for 10 min prior to incubation with DNP-HSA. Each datum represents the mean ± S.E. of six independent experiments. *P < 0.05; significantly different from the saline value.
layer membrane by causing a perturbation of the membrane. This result indicates that the membrane permeability increase may be an essential trigger for the release of the mediator from mast cells. In this sense, anti-allergic agents having a membrane stabilizing action may be desirable. VRFE might act on the lipid bilayer membrane affecting the prevention of the perturbation being induced by compound 48/80. The VRFE-administered rats are protected from IgE-mediated allergic reaction. The mechanism of protection against anti-DNP IgE, while not clear at present, may be evidenced only in particular conditions. More studies will be needed to define the reasons. Our data showed that VRFE inhibited anti-DNP IgE-induced TNF-α production. The effect of VRFE on mast cell cytokine production in vivo and the relative importance of mast cells as a source of TNF-α during inflammatory and immune responses are important areas for future studies.

In conclusion, the results obtained proved that VRFE inhibited the immediate-type allergic reactions in vivo and in vitro in a murine model. Further work should address the possibility that VRFE may also be active in the inhibition of human mast cell degranulation and, therefore, in the treatment of human allergic disorders. It will be necessary to investigate further the composition (protein, carbohydrate, etc.).

Acknowledgements

This work was supported by Woosuk University in 1999.

References


Antiviral activities of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* alone and in combinations with interferons

Young-So Kim, Seong-Kug Eo, Ki-Wan Oh, Chong-kil Lee, Seong-Sun Han *

College of Pharmacy, Chungbuk National University, Cheongju 361-763, South Korea

Received 5 November 1999; accepted 10 May 2000

Abstract

To investigate antiviral activity, an acidic protein bound polysaccharide (APBP) was isolated from carpophores of *Ganoderma lucidum*. This brownish APBP was isolated from water soluble substances of the carpophores by activity-guided isolation method. APBP was tested for its antiviral activity against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) by plaque reduction assay in tissue culture. APBP showed potent antiviral activity against HSV-1 and HSV-2 in Vero cells at its 50% effective concentration (EC50) of 300 and 440 μg/ml, respectively. APBP had no cytotoxicity on Vero cells at a concentration of 1 \times 10^4 μg/ml. APBP exhibited a potent antiviral activity with selectivity index (SI) of more than 22.73. The combined antiviral effects of APBP with protein antiviral agents, interferon alpha (IFN alpha) and interferon gamma (IFN gamma), were examined on the multiplication of these two strains of herpesviruses in Vero cells by the combination assay. The results of combination assay were evaluated by the combination index (CI) that was calculated by the multiple drug effect analysis. The combinations of APBP with IFN alpha on HSV-1 and HSV-2 showed more potent synergistic effects with CI values of 0.30–0.62 for 50–90% effective levels than those of APBP with IFN gamma with CI values of 0.65–1.10. These results suggest the possibility of developing APBP as a new antiviral agent. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Ganoderma lucidum*; Antiherpetic activity; Herpes simplex viruses; Plaque reduction assay; Combination index (CI)

1. Introduction

*Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae), basidiomycetous fungi has been used to treat various human diseases such as hepatitis, hypertension, arthritis, bronchitis, and tumorigenic diseases in oriental folk medicine (Kim and Kim, 1990).

Investigations into the biologically active components of the carpophores of cultured mycelia of *G. lucidum* have shown that this mushroom has various biological activities. *G. lucidum* was reported to contain some intensive bitter components including lucidenic acid A, B, C, D, E,
lucidone A, and ganoderic acid B and C (Nishitoba et al., 1985a,b). *G. lucidum* has also been reported to contain polysaccharides and protein bound polysaccharides which have antitumor (Kim et al., 1980; Miyazaki and Nishijima, 1981) and antihypertension activities (Park et al., 1987) and decrease the blood glucose level (Hikino et al., 1985). And it was reported that the water soluble substances of carpophores of *G. lucidum* do not have any serious and lethal effects on the acute toxicity when administered in vivo (Kim et al., 1986).

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are responsible for a broad range of human infectious diseases. Moreover, HSV infections were reported to be recognized as a risk factor for human immunodeficiency virus (HIV) infection (Hook et al., 1992). HSV-2 is also known as oncogenic virus which has the ability to convert cells into tumor cells (Lapucci et al., 1993). The various drugs with a clinically relevant activity against HSV infections include interferons (IFNs), acyclovir (ACV), vidarabine (ara-A), ganciclovir (DHPG) and phosphonoformic acid (foscarnet, PFA). However, these drugs have some undesirable complications and also induced emergence of drug-resistant viruses (Coen, 1991). Therefore, it is necessary to develop the new antitherpetic agents, and combination therapy with currently available drugs is attractive.

In the previous study on antitherpetic activities of water soluble substances isolated from *G. lucidum*, it was found that the carpophores of *G. lucidum* contain protein bound polysaccharides that can inhibit the HSV multiplication in vitro (Eo et al., 1999). In this study, the antitherpetic activity of an acidic protein bound polysaccharide (APBP) isolated from the carpophores of *G. lucidum*, and combined antitherpetic effects of APBP with interferon alpha (IFN alpha) or interferon gamma (IFN gamma) is investigated.

2. Materials and methods

2.1. Materials and reagents

Artificially grown carpophores of *G. lucidum* (Fr.) Karst (Ganodermataceae) were purchased from a local herbal drug store and authenticated by Dr Byung Kak Kim, Department of Microbial Chemistry, Seoul National University. A voucher specimen (No. Cpm 605) has been deposited at the Medicinal Plants Herbarium of the college. Eagle’s minimum essential medium (MEM), trypsin, penicillin, streptomycin, and amphotericin B were purchased from Gibco BRL (Gaithersburg, MD). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet were purchased from Sigma (St. Louis, MO). Recombinant human IFN alpha and recombinant human IFN gamma used in combination assays were obtained from Boehringer Mannheim (Mannheim, Germany) and Genzyme (Cambridge, MA), respectively, and calibrated by standards generously supplied from National Institute of Health (NIH), USA. Vero cell (African green monkey kidney cell, ATCC CCL 81), HSV-1 F strain ATCC VR-733 and HSV-2 G strain ATCC VR-734 were obtained from the American Type Culture Collection (Rockville, MD).

2.2. Activity-guided isolation of APBP

The carpophores of *G. lucidum* (500 g) were extracted with hot water for 8 h. The water extract was concentrated to one tenth of the original volume, and three volumes of ice cold EtOH added to precipitate the high molecular weight components. Then the sample was allowed to stand overnight at 4°C. It was centrifuged and the obtained precipitate was lyophilized. The lyophilized GLhw (3.88 g) was brownish powder with high molecular weight component of water soluble substances. The GLhw, a brownish powder was separated into neutral and acidic fractions by using the DEAE-cellulose column chromatography. The GLhw (3 g) was applied on to the DEAE-cellulose (Cl-form, Sigma, St. Louis, MO) column (bed volume = 50 ml). It was eluted with H2O (pH 7.2) followed by 2 M NaCl. The anthrone (620 nm) and Lowry–Folin tests (540 nm) were performed against each fraction. The fraction shown by the positive anthrone and Lowry–Folin tests was designated as neutral protein bound polysaccharide (NPBP, white powder, 550 mg). The other brown-
Table 1

Antiherpetic activity of APBP, interferon (IFN) alpha and IFN gamma on HSV-1 and HSV-2 in Vero cells by plaque reduction assay

<table>
<thead>
<tr>
<th>Antiherpetic Substances</th>
<th>CC50</th>
<th>EC50</th>
<th>SI&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HSV-1</td>
<td>HSV-2</td>
<td>HSV-1</td>
</tr>
<tr>
<td>APBP (&lt;i&gt;μg/ml&lt;/i&gt;)</td>
<td>&gt;10 000</td>
<td>300.04</td>
<td>440.03</td>
</tr>
<tr>
<td>IFN alpha (IU/ml)</td>
<td>898.19</td>
<td>101.62</td>
<td>1033.59</td>
</tr>
<tr>
<td>IFN gamma (IU/ml)</td>
<td>1033.59</td>
<td>402.41</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> APBP, acidic protein bound polysaccharide isolated from <i>Ganoderma lucidum</i>.

<sup>b</sup> Selectivity index (SI) = CC50/EC50.

<sup>c</sup> HSV-1, herpes simplex virus type 1 F strain.

<sup>d</sup> HSV-2, herpes simplex virus type 2 G strain.

ish and acidic fraction which showed positive anthrone and Lowry–Folin tests was designated as APBP (850 mg).

2.3. Cells and viruses

Vero cells were cultured with MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and were subcultured twice a week. Virus stocks were prepared in Vero cell cultures and stored at −70°C. Virus titer was determined by using plaque assay.

2.4. Cytotoxicity assay

For cytotoxicity assay, Vero cells were seeded in 96 well plates (Falcon, NJ) at an initial density of 3.5 x 10<sup>4</sup> cells per well. After incubation of the cells for 16–18 h at 37°C, various concentrations of APBP were added, and the incubation was continued for 48 h. Viable cell yield was determined by MTT reduction assay according to reported procedure (Scubiero et al., 1988). The cytotoxicity was expressed as the 50% cytotoxic concentration (CC<sub>50</sub>) which is the concentration of substances to inhibit the growth of cells up to 50% by regression analysis.

Fig. 1. Inhibitory effects of plaque formation of herpes simplex virus type 1 (HSV-1) in Vero cells by acidic protein bound polysaccharide (APBP), interferon (IFN) alpha (A) and IFN gamma (B) alone, and their combinations at a fixed ratio of 2:1. Results are expressed as percent with respect to virus control (VC) group. Each value is the mean ± S.D. of quadruplicate determination. * Not significantly effective than VC; <i>P</i> > 0.05 (Student t-test).
Table 2
Median effective concentrations and CI values of combinations of APBP$^a$ with interferon (IFN) alpha and IFN gamma on the plaque formation of herpes simplex virus type 1 (HSV-1) in Vero cells

<table>
<thead>
<tr>
<th>Materials</th>
<th>Parameters$^b$</th>
<th>CI at f(a) of$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m</td>
<td>EC$_{50}$</td>
</tr>
<tr>
<td>APBP ($\mu$g/ml)</td>
<td>1.43</td>
<td>350.08</td>
</tr>
<tr>
<td>IFN alpha (IU/ml)</td>
<td>1.30</td>
<td>898.19</td>
</tr>
<tr>
<td>IFN gamma (IU/ml)</td>
<td>1.43</td>
<td>1033.59</td>
</tr>
<tr>
<td>APBP/IFN alpha (2:1)</td>
<td>1.61</td>
<td>180.49/90.25</td>
</tr>
<tr>
<td>APBP/IFN gamma (2:1)</td>
<td>1.43</td>
<td>334.12/167.06</td>
</tr>
</tbody>
</table>

$^a$ APBP, acidic protein bound polysaccharide isolated from *Ganoderma lucidum*.

$^b$ m is the slope, EC$_{50}$ is the median effective concentration, and r is the correlation coefficient as determined from the median-effect plot.

$^c$ CI means the combination index obtained for three different effect levels, 50, 70, and 90% inhibition (EC$_{50}$, EC$_{70}$, and EC$_{90}$) of the virus plaque formation. Values of <1, 1, and >1, indicate synergism, additivity, and antagonism, respectively. CI values were determined under mutually exclusive assumptions.

2.5. Antitherpetic activity assay

Antitherpetic activity was evaluated by plaque reduction assay (Shigeta et al., 1992). Host cell monolayers grown in 12 well plates (Falcon, NJ) were infected with about 150 pfu of virus per well in the absence or presence of various concentrations of APBP. After 1 h adsorption, agar overlay medium containing APBP at various concentrations was overlaid. After 1–3 days of incubation at 37°C, virus plaques were counted. The degree of inhibition was expressed as the 50% effective concentration (EC$_{50}$) which was calculated as the concentration of substance required to reduce virus plaque by 50% using regression analysis. Antitherpetic activity for each substance was evaluated by selectivity index (SI) which was calculated by dividing the CC$_{50}$ by EC$_{50}$.

2.6. Combination assay

The combination assay was essentially performed according to the published method (Tachedjian et al., 1991) with some modification. Vero cells at 3.5 × 10^5 cells/well were incubated into 12 well culture plate. After incubation at 37°C for 18 h, a confluent cell monolayer was generally obtained. At this time a cell monolayer was preincubated with IFN alpha and IFN gamma in the concentrations range of 10–200 IU/ml or 50–1000 IU/ml at 37°C for 1 h, and washed with PBS (pH 7.4). A cell monolayer was infected with virus alone and virus plus APBP in the concentrations range of 100–2000 $\mu$g/ml, and the plates were incubated at 37°C for 1 h with intermittent rocking at 15 min intervals. Then the plates were overlaid with agar overlay medium containing APBP in a constant ratio compared with concentrations of IFN alpha and IFN gamma. The above plates were incubated at 37°C until the formation of plaques. The formed virus plaques were counted.

2.7. Calculation and analysis of drug interactions

For the determination of synergistic or antagonistic drug interactions using multiple drug analysis procedure (Schinazi et al., 1986), data was expressed as the fraction affected relative to the untreated control cultures. Multiple drug effect analysis involves plotting the results obtained for each drug alone, or when combined at multiple fixed-ratio drug concentrations, in the form of a dose-effect curve defined by the median effect equation $f(a)/f(u) = (C/C_m)^m$. The $f(a)$ and $f(u)$ are the fractions affected and unaffected, respectively, by the concentration $C$. $C_m$ is the concen-
tration required to produce the median effect (i.e. 50% effective dose), and \( m \) represents the sigmoidicity of the curve. If the slopes \( (m) \) of the dose-effect curves for each drug alone and in combination are all parallel, such drugs are said to be mutually exclusive (similar mode of action). Conversely, a mutually nonexclusive case (different mode of action) is defined by parallel gradient for each drug alone, and with the gradient for the drug combinations being non-parallel. A combination index (CI) can be calculated for either mutually exclusive or nonexclusive assumptions. The CI values less than 1.0 indicate synerigism, and CI values greater than 1.0 represent antagonism, and CI values equal to 1.0 indicate additive effect.

For the analysis of combinations of APBP with IFN alpha and IFN gamma by multiple drug effect analysis, only data with high linear correlation coefficients \( (r > 0.8) \) as determined by the median effect plots were used in this analysis.

3. Results and discussion

In an attempt to find antiherpetic substances to reduce the adverse side effects associated with long term therapy and limit the emergence of resistant virus, APBP was isolated from the car-

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**Fig. 2.** Inhibitory effects of plaque formation of herpes simplex virus type 2 (HSV-2) in Vero cells by acidic protein bound polysaccharide (APBP), interferon (IFN) alpha (A) and IFN gamma (B) alone, and their combinations at a fixed ratio of 10:1 (APBP:IFN alpha) or 2:1 (APBP:IFN gamma). Results are expressed as percent with respect to virus control (VC) group. Each value is the mean \( \pm \) S.D. of quadruplicate determination. * Not significantly effective than VC; \( P > 0.05 \) (Student \( t \)-test).

**Fig. 3.** Median-effect plots for the inhibition of plaque formation of herpes simplex virus type 1 (HSV-1) by acidic protein bound polysaccharide (APBP), interferon (IFN) alpha (A) and IFN gamma (B) alone, and their combinations at a fixed ratio of 2:1.
pophores of *G. lucidum*. Antiviral activity of APBP against HSV-1 and HSV-2 was evaluated in Vero cells by plaque reduction assay. The inhibitory effects of APBP on plaque formation of HSVs in Vero cells are given in Table 1. APBP showed potent antiviral activity against HSV-1 and HSV-2 in Vero cells at its EC50 of 300 and 440 µg/ml, respectively. APBP had no cytotoxicity on Vero cells at a concentration of 1 × 10^4 µg/ml. Therefore, APBP exhibits a potent antiviral activity with SI of more than 22.73.

In a previous study, APBP was shown to contain mainly polysaccharide (approximately 40.6%) and protein (approximately 7.80%) as identified by anthrone and Lowry–Folin tests, respectively. Further APBP showed the usual molar ratio (C:H:O = 1:2:1) of carbohydrates as determined by elemental analysis.

It is known that the antiviral activities of polysaccharides are linked to the anionic features of the molecules. They inhibit at the very early stages of viral infections such as attachment and penetration (Shannon, 1984), and increase with the molecular weight or the degree of sulfation (Witvrouw et al., 1994). Therefore, the antinfective activity of APBP would be expected to increase further by sulfation or partial digestion.
The identification of the possible mode of antiherpetic activity and the molecular entity of APBP are currently in progress.

The various reasons for combination chemotherapy for viral infections include synergy of antiviral effects, antagonism of toxicities, distribution of toxicities among organ systems, prevention of emergence of resistant variants, and enhancement of immune functions (Schinazi et al., 1986). To determine whether any antiviral synergism between APBP and antitherpetic agents, combined effects of APBP with IFN alpha and IFN gamma were examined on the plaque formation of HSV-1 and HSV-2 in Vero cells.

The inhibitory effects of plaque formation of HSV-1 in Vero cells by APBP, IFN alpha and IFN gamma alone, and their combinations are presented in Fig. 1. APBP, IFN alpha and IFN gamma alone showed a concentration-dependent inhibition of plaque formation of HSV-1 in Vero cells. When combined, the drugs represented a greater inhibition of plaque formation of HSV-1 at lower concentrations. The CI values with IFN alpha and IFN gamma on HSV-1 for 50, 70 and 90% effect levels are given in Table 2 and CI values for combinations of APBP with IFN alpha or IFN gamma on HSV-1 in Vero cells corresponding to fraction affected are shown in Fig. 5. Since the slopes ($m$) of the dose-effect curves for each drug alone and in combination was parallel (Fig. 3), the CI values were calculated under mutually exclusive assumptions. The CI values were in the range of 0.50–0.62 for a combination of APBP with IFN alpha, and in the range of 1.09–1.10 for a combination of APBP with IFN gamma. Therefore, the combinations of APBP with IFN alpha showed synergistic effects on the plaque formation of HSV-1 in Vero cells, whereas the combination of APBP with IFN gamma showed antagonistic effect.

The inhibitory effects of plaque formation of HSV-2 by APBP, IFN alpha and IFN gamma alone, and their combinations are presented in Fig. 2. APBP, IFN alpha and IFN gamma alone showed concentration-dependent reduction of plaque formation of HSV-2. The combinations of APBP with IFN alpha or IFN gamma showed a potent inhibition than when tested for individual drugs. Because the plots of both compounds and their combinations were all parallel (Fig. 4), the CI values were determined by using mutually exclusive assumptions. The CI values of APBP with IFN alpha or IFN gamma on HSV-2 for 50, 70 and 90% effect levels were given in Table 3. CI values were in the range of 0.30–0.31 for a combination of APBP with IFN alpha, and in the range of 0.65–0.76 for a combination of APBP with IFN gamma. Therefore, the combination of APBP with IFN alpha or IFN gamma on HSV-2 showed a synergism.

### Table 3

Median effective concentrations and CI values of combinations of APBP\(^a\) with interferon (IFN) alpha and IFN gamma on the plaque formation of herpes simplex virus type 2 (HSV-2) in Vero cells

<table>
<thead>
<tr>
<th>Materials</th>
<th>Parameters(^b)</th>
<th>CI at f(a) of: (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$m$</td>
<td>EC(_{50})</td>
</tr>
<tr>
<td>APBP (µg/ml)</td>
<td>1.28</td>
<td>480.72</td>
</tr>
<tr>
<td>IFN alpha (IU/ml)</td>
<td>1.20</td>
<td>101.62</td>
</tr>
<tr>
<td>IFN gamma (IU/ml)</td>
<td>1.18</td>
<td>402.41</td>
</tr>
<tr>
<td>APBP/IFN alpha (10: 1)</td>
<td>1.24</td>
<td>97.61/9.76</td>
</tr>
<tr>
<td>APBP/IFN gamma (2: 1)</td>
<td>1.36</td>
<td>228.41/114.20</td>
</tr>
</tbody>
</table>

\(^a\) APBP, acidic protein bound polysaccharide isolated from *Ganoderma lucidum*.

\(^b\) $m$ is the slope, EC\(_{50}\) is the median effective concentration, and $r$ is the correlation coefficient as determined from the median-effect plot.

\(^c\) CI means the concentration index obtained for three different effect levels, 50, 70, and 90% inhibition (EC\(_{50}\), EC\(_{70}\), and EC\(_{90}\)) of the virus plaque formation. Values of <1, 1, and >1, indicate synergism, additivity, and antagonism, respectively. CI values were determined under mutually exclusive assumptions.
In conclusion, APBP showed potent antiviral activity against HSV-1 and HSV-2 in Vero cells with SI of more than 22.73. The combination of APBP with IFN alpha on HSV-1 showed synergistic effect, whereas the combination with IFN gamma showed an antagonistic effect. On the contrary, the combinations of APBP with IFN alpha or IFN gamma on HSV-2 showed synergistic effects. Based on these studies, APBP may be able to use both as a potential antitherpetic and combination therapy agents that can permit a significant reduction in the dosage of the toxic antitherpetic agents without compromising antiviral activity.

Acknowledgements

This work was supported by a grant from the Regional Center for Health and Life Science, Chungbuk National University.

References


Variation of the antimicrobial activity of *Pseudognaphalium vira vira* (Asteraceae): isolation and X-ray structure of *ent*-3β-hydroxy-16-kauren-19-oic acid

Marcos Caroli Rezende a,*, Alejandro Urzua a, Adailton J. Bortoluzzi b, Loretta Vásquez a

a Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo 33, Santiago, Chile
b Departamento de Química, Universidade Federal de S. Catarina, Florianópolis, SC, Brazil

Received 29 October 1999; received in revised form 22 February 2000; accepted 10 April 2000

Abstract

The antimicrobial properties of the resinous exudate from twigs and leaves of a population of *Pseudognaphalium vira vira* were re-examined against five Gram positive bacteria. The observed decrease in the antimicrobial activity might reflect a change in the diterpenoid composition of the exudate. The title compound 2 was isolated in substantial amount, together with *ent*-16-kauren-19-oic acid (1), and its structure determined by X-ray diffraction analysis. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Hydroxykaurenoic acid; Antimicrobial activity; X-ray structure

1. Introduction

Kauranes constitute a widespread class of diterpenes with a rigid tetracyclic skeleton. In a recent review on these compounds, examples of antimicrobial, antiparasitic, insect antifeedant, anti-HIV and antiinflammatory activities have been reported for different kauranes (Guisalberti, 1997). *Ent*-16-kauren-19-oic acid (1) is an ubiquitous member of this family, exhibiting most of these interesting biological properties.

We had previously reported the isolation of kaurenoic acid from the resinous exudates of four Chilean species of *Pseudognaphalium* (Mendoza et al., 1997; Mendoza and Urzua, 1998). The antimicrobial activity of these plants, employed in folk medicine as antiseptic agents and in the treatment of bronchial illnesses, correlated well with the presence of compound 1 in *P. vira vira* and *P. robustum* (Mendoza et al., 1997). Kaurenoic acid was shown to be active against five Gram positive...
bacteria (Bacillus cereus, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus and Clavibacter michiganensis) with MIC values that ranged from 16 to 125 µg ml⁻¹. By contrast, the 3β-hydroxylated derivative of 1, ent-3β-hydroxy-16-kauren-19-oic acid (2), was much less active, or even inactive, against the same micro-organisms (Scheme 1).

Compound 2 had been isolated in 1994 from P. cheirantifolium and P. heterotrichium, but not from P. vira vira, which yielded compound 1 as the only kaurane diterpene (Urzua et al., 1995). The latter species is by far the most widely employed Pseudognaphalium in Chilean folk medicine. To our surprise, samples of P. vira vira collected in the same place, but four years later, proved to be much less active against the same microorganisms. An analysis of the exudate of these samples yielded, in addition to compound 1, a substantial amount of the hydroxylated derivative 2. This intriguing observation suggested to us the idea that both compounds are metabolized by these plants in relative concentrations which depend to some degree on climatic conditions. In addition, we decided to look more closely at both structures, in the search of a possible explanation for the reduction in activity of the kaurenoic acid substrate, when a hydroxy group is introduced near the carboxylic group of 1.

In the present communication we describe the isolation of ent-3β-hydroxy-16-kauren-19-oic acid (2) from the exudate of P. vira vira and present the structure of this compound as determined by X-ray diffraction analysis. This structural information should be a piece of evidence in elucidating the mode of action of kaurenoic acid and related antibacterial terpenoids isolated from a variety of medicinal plants.

2. Materials and methods

2.1. Plant material

Aereal parts of P. vira vira (SGO133615-98) were collected during the flowering season, in September 1998, between Zapallar and Papudo (V Región Chile, 32°30′ S, 71°30′ W). Voucher specimens were deposited in the Herbarium of the National History Museum, Santiago, Chile.

2.2. Isolation of pure compounds from the resinous exudate

The resinous exudate of P. vira vira was obtained by dipping 21 kg of the fresh plant in cold CH₂Cl₂ for 15–20 s, and concentrating in a rotary evaporator the organic extracts to a sticky residue which weighed 55 g. This material was fractioned by column chromatography on silica gel, employing n-hexane with increasing amounts of ethyl acetate as eluent. The fractions were monitored by thin-layer chromatography on silica gel, using the systems hexane–EtOAc (3:1), CHCl₃–MeOH (99:1) and CHCl₃–MeOH (95:5) as eluents. The isolated ent-16-kauren-19-oic acid (1) (7.8 g) and ent-3β-hydroxy-16-kauren-19-oic acid (2) (6.2 g) were identified by direct comparison with authentic samples (FTIR, TLC, HPLC), previously isolated from other Pseudognaphalium species (Urzua et al., 1995; Mendoza and Urzua, 1998). The ent-3β-hydroxy-16-kauren-19-oic acid (2) was crystallized from petroleum ether, m.p. 215–216°C.

2.3. Antimicrobial activity determination

The antimicrobial activity of the CH₂Cl₂ extracts against Gram positive bacteria was determined by the agar overlay method (Mayr-Harting
Table 1

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>MIC (μg ml⁻¹)</th>
<th>Sample I</th>
<th>Sample II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>B. cereus</strong></td>
<td>16</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>31</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td><strong>B. subtilis</strong></td>
<td>250</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td><strong>M. luteus</strong></td>
<td>125</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><strong>C. michiganensis</strong> subsp.</td>
<td>63</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td><em>michiganensis</em></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Data from Mendoza et al. (1997).

*b* This work.

e t al., 1972), following the protocol described previously (Mendoza et al., 1997). The extracts were tested against five different microorganisms: *B. cereus* (NAS 569), *S. aureus* (ATCC 6538p), *B. subtilis* (ATCC 6633), *M. luteus* (ATCC 9341) and *C. michiganensis* subsp. *michiganensis* (Cmm 623).

2.4. X-ray diffraction analysis of compound 2

A colorless crystal with the dimensions 0.50 × 0.50 × 0.07 mm³ was selected for X-ray analysis. The crystal was mounted on an Enraf-Nonius CAD-4 diffractometer with graphite-monochromatized Mo K\(\alpha\) radiation at room temperature. Cell parameters: \(a = 9.715(2)\) Å, \(b = 17.329(4)\) Å, \(c = 10.429(2)\) Å, \(\beta = 93.63(3)°\) and \(V = 1752.2(6)\) Å³ were obtained by the least-squares refinement of 25 reflections in the range 10.04 < \(\theta\) < 18.12. Unique reflections (3531) (\(R_{int} = 0.0286\)) were collected in the range 2.28 < \(\theta\) < 25.96 using the \(\omega - 2\theta\) technique with three reflections for intensity control and with two reflections for orientation control. No significant intensity decay was observed during the data collection. The HELENA program (Spek, 1996) was used to perform the data reduction process. The structure was solved by direct methods and was refined by the full-matrix least-squares method using SHELXS97 (Sheldrick, 1997a) and SHELXL97 (Sheldrick, 1997b) computer programs, respectively. Hydrogens attached to C atoms were added at their calculated positions and included in the structure factor calculations using a riding model. Hydrogen atoms of the alcohol and acid groups were located in the Fourier-difference map and refined with no positional constraints, but with isotropic thermal parameters fixed at 1.2 times the equivalent isotropic parameter of the attached O atoms. All non-hydrogen atoms were refined anisotropically.

Final indices: \(R\) (on \(F\)) = 0.0406, \(wR\) (on \(F^2\)) = 0.1006 and goodness of fit = 1.046.

3. Results and discussion

In Table 1 are listed the minimal inhibitory concentrations (MIC) obtained in the present study for the five Gram positive bacteria, together with the corresponding MIC values obtained previously (Mendoza et al., 1997). Notice that the MIC values were determined for the same microorganisms, following the same procedure for both populations of *P. vira vira*, collected in the same location, within a 4-year interval.

It is seen that the extracts from the sample collected in 1998 consistently showed a diminished activity against all five bacteria, compared with the extracts from the plant collected in 1994. An analysis of the diterpenoid contents of the plant extracts revealed a considerable variation from those reported previously. Only kaurenoic acid 1 had been isolated previously from the resinous exudates of *P. vira vira*. In the present sample, compound 1 and its hydroxylated derivative 2 were present in the resinous exudates in nearly a 1:1 proportion. This might explain the diminished antibacterial activity of the studied extracts, since compound 2, isolated from other *Pseudognaphalium* species, had proved much less active than kaurenoic acid 1 against the same bacteria (Mendoza et al., 1997). We may rule out an alternative explanation, based on variations in the content of other antibacterial compounds, such as polyynes and their sulfur derivatives, eventually present in the extracts. Previous gas chromatographic analyses did not detect polyynes in the resinous exudates of four Chilean *Pseudognaphalium* species, including *P. vira vira* (Men-
doza and Urzua, 1998). This was reinforced by a gas-chromatographic/mass-spectrometric analysis of the exudate extracts of the 1998 *P. vira vira* sample. In addition to compounds 1 and 2 and traces of some monoterpenes, only saturated, normal alkanes in the range C\textsubscript{27}–C\textsubscript{35} were identified, none of the latter exhibiting antimicrobial activity.

In trying to gain some insight into the structural requirements for the antibacterial activity of kaurenoic acid and its derivatives, we next resorted to X-ray diffraction analysis to obtain the structure of the isolated hydroxykaurenoic acid 2.

The crystal lattice of acid 2 belongs to the monoclinic system and its space group was determined to be P\textsubscript{2}1 (No. 4). Two independent molecules of compound 2 were found in the asymmetric unit, depending on which oxygen atom of the carboxylic group hydrogen-bonds to the adjacent \(\alpha\)-hydroxy group. An ortep projection of both structures is shown in Fig. 1. Extensive inter- and intramolecular hydrogen-bonding between the –OH and the –CO\textsubscript{2}H groups is observed, as can be seen in Fig. 2, which reproduces the arrangement of three molecules of compound 2 in the crystal cell. These hydrogen-bonds are an important piece of evidence in discussing the structural requirements for the activity of kaurenoic acids and related antibacterial compounds.

Comparison of the structure of 1 with that of salvic acid and its acetate, diterpenoids isolated from the exudate of a resinous herb employed in

![Fig. 1. Ortep projections of the two molecules found in the asymmetric unit of the monoclinic crystal of ent-3\(\beta\)-hydroxy-16-kauren-19-oic acid (2). For the sake of an easier visualization, only a few relevant hydrogen atoms are shown.](image-url)
Chilean folk medicine (Urzua et al., 1997, 1998), reveals common structural features, such as a rigid, polycyclic hydrophobic skeleton and a hydrophilic region possessing an acidic carboxylic proton.

Studies on the antibacterial action of kaurenoic acid 1 showed that this compound is active against Gram positive, but inactive against Gram negative bacteria (Alarcon et al., 1997). The same was true of salvic acid and its acetate (Urzua et al., 1998). In contrast with vancomycin, a reference antibiotic that caused lysis of B. cereus only after 60 min of incubation, addition of kaurenoic acid 1 produced almost immediate cell lysis of that microorganism (Alarcon et al., 1997). These observations point to a mechanism based on the disruptive action on the cell membrane by kaurenoic acid. The fact that the acid is unable to penetrate the negatively-charged external membrane of Gram negative bacteria may be interpreted as arising from a repulsion between the negative surface and the ionized, negatively charged carboxylate group of 1. However, the same carboxylic group should be important in the cell lysis of Gram positive bacteria. The diminished activity of this kaurane derivative when the carboxylic group is esterified (Mitscher et al., 1983; Guisalberti, 1997) leads to this conclusion. It seems reasonable to assume that, for an effective interaction of the diterpenoid with the bacterial cell membrane, the pharmacofore should possess a rigid, polycyclic hydrophobic skeleton, coupled with a hydrogen-bond-donor carboxylic group. The latter could well interact with a hydrogen-bond-acceptor site of the membrane. Elimination of this donor–acceptor interaction by esterification should thus diminish the activity of the diterpenoid. This is reinforced by the observation that derivative 2, with an –OH group introduced near the carboxylic centre, also exhibits reduced antibacterial activity. In this case, however, the acidic proton of the carboxylic group is not suppressed by esterification, but becomes engaged in intramolecular hydrogen-bonds with the neighbouring hydroxyl group, with the result that the –CO\textsubscript{2}H group is now less available for donor–acceptor interactions with the membrane site. The existence of extensive intramolecular hydrogen-bonds in 2, depicted in Fig. 2, supports this model.

In conclusion, the presence of hydroxykaurenoic acid 2 as a major component of the exudate of a sample of P. viridis collected in 1998 departs from previous observations made on samples collected in the same location, but 4 years earlier, which contained only kaurenoic acid 1. The possibility that this variation might be caused by severe climatic changes in the past years, brought about by the phenomenon of El Niño in Chile, seems to us worth investigating.

The fact that compound 2 is a less active antibacterial agent than acid 1 might explain the diminished activity of the 1998 plant extracts, when compared with those of P. viridis samples collected 4 years later.

A comparison of the structures of diterpenoids 1 and 2 stresses the importance of a free –CO\textsubscript{2}H as a requisite for the antibacterial activity of these diterpenoids. Suppression of the acidic hydrogen by esterification, or reduction of its availability by intramolecular hydrogen-bonding with an OH group, has the effect of decreasing this activity.
Further structure-activity-relationship studies with kaurenoic and salvic acid derivatives to verify these hypotheses are in progress in our laboratories.

Supplementary material such as tables of thermal parameters, complete listing of bond distances and bond angles and full details of the data collection and refinement of the structure are available at the CCDC, reference number 136831.

Acknowledgements

We are grateful to Fondecyt (Projects 2980043 and 1990209), to Conicyt, for a grant to LVF, and to Dicyt (USACH) for supporting this work.

References


Short communication

Evaluation of the antiulcerogenic activity of friedelan-3β-ol and friedelin isolated from *Maytenus ilicifolia* (Celastraceae)

Carmen Lucia Queiroga a,*, Guilherme Faria Silva a, Patrícia Corrêa Dias b,c, Ana Possenti b, João Ernesto de Carvalho b

a Divisão de Fitoquímica, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas-CPQBA, Universidade Estadual de Campinas-UNICAMP, CP 6171, CEP 13081-970-Campinas, São Paulo, Brazil

b Divisão Farmacologia e Toxicologia, Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agrícolas-CPQBA, Universidade Estadual de Campinas-UNICAMP, CP 6171, CEP 13081-970-Campinas, São Paulo, Brazil

c Departamento de Farmacologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, CP 6111, CEP 13081-970 Campinas, São Paulo, Brazil

Received 20 July 1999; received in revised form 28 March 2000; accepted 10 April 2000

Abstract

An easy methodology for triterpene isolation is shown. Evaluation in rats for antiulcer activity of friedelan-3β-ol 1 and friedelin 2. The two triterpenes isolated from the leaves of *Maytenus ilicifolia*, did not decrease gastric ulcers when tested on indometacine induced ulcer model in rats. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Maytenus ilicifolia*; Triterpenes; Friedelan-3β-ol; Friedelin; Gastric ulcers

1. Introduction

Numerous compounds isolated from the *Maytenus ilicifolia* Mart. ex. Reiss (Celastraceae) have been tested for their antitumoral activity (Itokawa et al., 1991; Shirota et al., 1994), however, the infusion of the leaves from the *Maytenus ilicifolia* and/or *Maytenus aquifolia* have been used in Brazil by the folk medicine for their antacid and antiulcerogenic effects (Souza-Formigoni et al., 1991). The antiulcer properties have been attributed to triterpenes (Vilegas and Lanças, 1994), but these data have not been confirmed. The goal was confirming the effects of these triterpenes to subsequent selection of *M. ilicifolia* plants cultivated in CPQBA. The present paper shows an easy methodology for isolation of friedelan-3β-ol 1 and friedelin 2 and their effects on gastric ulcers.

* Corresponding author.
E-mail address: queiroga@cpqba.unicamp.br (C.L. Queiroga).
2. Materials and methods

2.1. Extraction and preliminary isolation

Leaves of *M. ilicifolia* were collected in the experimental field of CPQBA-UNICAMP. A sample of the material was deposited in the herbarium of CPQBA–UNICAMP (Register No. 255). Two hundred and fifty grams of dried powdered leaves were extracted with hexane (1.5 l) for 14 h, following a reextraction with hexane (1.0 l) for 12 h. After filtration, the extracts were combined, concentrated in a vacuum evaporator at 40°C and treated with active charcoal. The crude hexane extract (5.24 g) was dissolved in chloroform (40 ml) and acetone added (40 ml). Immediately occurring precipitation. The mixture was maintained at 0°C for 24 h and afterwards filtered. The precipitate was washed with cool acetone and dried to give a mixture of friedelan-3ß-ol 1 and friedelin 2 (0.99 g, 18.9% yield).

2.2. GC/MS

Analytical GC/MS was performed on a HP5890/5971 system fitted with SA-5 fused silica capillary column (30 m × 0.25 mm × 0.25 µm). The carrier gas was helium with flow rate 1 ml/min. Column temperature was programmed from 120°C (2 min)–5°C/min–240°C (40 min) (5). Injector and detector temperatures were 280 and 300°C. Injection volume, 1 µl solution 10 mg/ml (triterpenes in ethyl acetate).

2.3. 13C NMR

13C NMR spectra were obtained with a Brucker AC 300 P (75.4 MHz) or a GEMINI 300 (75.4 MHz, Varian) spectrometer. CDCl3 (77.0 ppm) was used as internal standard. The polarization transfer technique, DEPT (135 and 90), was used for the multiplicity determination.

2.4. Isolation

The mixture of friedelan-3ß-ol 1 and friedelin 2 was purified by flash chromatography on silica gel (230–400 mesh) using dichloromethane obtained the pure friedelan-3ß-ol 1. The enriched fraction with friedelin 2 was repurified by flash chromatography using hexane/ethyl acetate (98:2) affording pure friedelin 2. The identification was made on the basis of melting point, mass spectrum and NMR13C spectrum of pure friedelan-3ß-ol 1 and pure friedelin 2 (Gottlieb et al., 1985; Prakash et al., 1987; Mahato and Kundu, 1994; Carvalho et al., 1998).

2.4.1. Friedelan-3ß-ol 1

m.p. 280–282°. GC/MS: tR 48.61 min; MS m/z (%): 428 (M+, 10), 413 (13), 275 (37), 248 (16), 231 (35), 220 (29), 206 (31), 191 (25), 177 (28), 165 (78), 149 (27), 135 (33), 123 (62), 107 (53), 95 (100), 81 (78), 69 (95), 55 (78), 43 (36), 41 (38). 13C NMR (75.4 MHz, CDCl3) 15.48 (C1, CH2), 31.91 (C2, CH2), 71.87 (C3, CH), 48.86 (C4, CH), 37.48 (C5, Cquat.), 41.38 (C6, CH2), 17.18 (C7, CH3), 52.78 (C8, CH), 36.70 (C9, Cquat.), 61.00 (C10, CH), 35.16 (C11, CH2), 29.28 (C12, CH2), 37.93 (C13, Cquat.), 39.11 (C14, Cquat.), 30.25 (C15, CH2), 35.68 (C16, CH2), 29.62 (C17, Cquat.), 17.18 (C18, CH), 34.94 (C19, CH2), 27.79 (C20, Cquat.), 32.41 (C21, CH2), 38.87 (C22, CH2), 11.37 (C23, CH3), 16.04 (C24, CH3), 17.89 (C25, CH3), 18.26 (C26, CH3), 19.73 (C27, CH3), 31.78 (28, CH3), 34.66 (C29, CH3), 31.44 (C30, CH3).

2.4.2. Friedelin 2

m.p. 261–262°. GC/MS: tR 49.30 min; MS m/z (%): 426 (M+, 12), 273 (39), 246 (33), 231 (30), 218 (32), 207 (58), 179 (37), 163 (46), 149 (21), 135 (28), 123 (64), 109 (76), 95 (99), 81 (74), 69 (100), 55 (70), 43 (26), 41 (43). 13C NMR (75.4 MHz, CDCl3) 21.90 (C1, CH2), 41.17 (C2, CH2), 212.73 (C3, Cquat.), 57.90 (C4, CH), 41.79 (C5, Cquat.), 41.00 (C6, CH2), 17.89 (C7, CH3), 52.79 (C8, CH), 37.15 (C9, Cquat.), 59.20 (C10, CH), 35.72 (C11, CH2), 29.31 (C12, CH2), 38.00 (C13, Cquat.), 39.39 (C14, Cquat.), 32.52 (C15, CH2), 35.30 (C16, CH2), 29.67 (C17, Cquat.), 42.55 (C18, CH), 34.94 (C19, CH2), 27.79 (C20, Cquat.), 32.41 (C21, CH2), 38.87 (C22, CH2), 11.37 (C23, CH3), 16.04 (C24, CH3), 17.89 (C25, CH3), 18.26 (C26, CH3), 19.73 (C27, CH3), 31.78 (28, CH3), 34.66 (C29, CH3), 31.44 (C30, CH3).
2.5. Biological assays

2.5.1. Indomethacin induced ulcer

Male Wistar rats (200–250 g), fasted for 24 h, with free access to water, were divided in at least three groups according to the respective treatment employed (saline, ranitidine 50 mg/kg or cimetidine 100 mg/kg and sample in study in previously determined dose). After 30 min of oral treatment, indomethacin (30 mg/kg) was administered subcutaneously to all groups of animals, according to the methodology described by Morimoto et al. (1991). After 4 h, the animals were sacrificed and their stomachs were removed, and opened along the greater curvature. The ulcerative lesion index of each animal was calculated by adding the following values, according to methodology described by Gamberini et al. (1991). Loss of normal morphology (1 point), discoloration of mucosa (1 point), hemorrhages (1 point), petechial points (until 9; 2 points), petechial points (more than 10; 2 points), ulcers up to 1 mm (*n x 2 points), ulcers greater than 1 mm (*n x 3 points), perforated ulcers (*n x 4 points), * number of ulcers found.

3. Discussion

The antiulcer activity of ‘espinheira-santa’ leaves, *M. ilicifolia* and *M. aquifolia* (Celastraceae), is very widespread in Brazil. Some researchers attribute this activity to the triterpenes friedelan-3β-ol 1 and friedelin 2 isolated from extracts of *M. ilicifolia* leaves. The course of our research has been testing the antiulcerogenic activity of different *M. ilicifolia* extracts and GC/MS analysis of the hexane extract indicated the presence of triterpenes. An enriched fraction of triterpenes was obtained in sufficient amount for the biological tests. In the techniques used it was observed that liquid–liquid partition induced precipitation. Analysis by GC/MS of the precipitate indicated the presence of two major components whose mass spectrum allowed them to be identified as friedelan-3β-ol 1 and friedelin 2 (relative purity 74.6%, proportion 6.4:1).

The evaluation of the mixture of friedelan-3β-ol 1 and friedelin 2 against gastric ulcer using the indomethacin induced ulcer model indicated that these triterpenes have no antiulcerogenic activity effects (Table 1). To confirm the absence of activ-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>ULI (mean ± S.E.M.)</th>
<th>ULI inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>5</td>
<td>38.6 ± 11.7</td>
<td>–</td>
</tr>
<tr>
<td>Ranitidine</td>
<td>50.0</td>
<td>5</td>
<td>4.3 ± 1.6*</td>
<td>88.9</td>
</tr>
<tr>
<td>Mixture of 1 + 2</td>
<td>300.0</td>
<td>5</td>
<td>32.6 ± 8.4</td>
<td>–</td>
</tr>
</tbody>
</table>

* P < 0.05: Duncan’s test. ANOVA F(2,11) = 3.81, P < 0.05.

Table 2

Effect of oral administration of pure friedelan-3β-ol 1 obtained from leaves of *Maytenus ilicifolia*, on indomethacin induced ulcer model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>ULI (mean ± S.E.M.)</th>
<th>ULI inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>5</td>
<td>27.2 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>5</td>
<td>2.8 ± 0.5*</td>
<td>89.7</td>
</tr>
<tr>
<td>Friedelan-3β-ol</td>
<td>10.0</td>
<td>5</td>
<td>42.6 ± 11.8</td>
<td>–</td>
</tr>
<tr>
<td>Friedelan-3β-ol</td>
<td>30.0</td>
<td>5</td>
<td>44.4 ± 12.6</td>
<td>–</td>
</tr>
<tr>
<td>Friedelan-3β-ol</td>
<td>100.0</td>
<td>5</td>
<td>33.8 ± 8.0</td>
<td>–</td>
</tr>
</tbody>
</table>

* P < 0.05: Duncan’s test. ANOVA F(4,20) = 3.25, P < 0.05.
Table 3
Effect of oral administration of pure friedelin 2, isolated from leaves of *Maytenus ilicifolia*, on indomethacin induced ulcer model

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>ULI (mean ± S.E.M.)</th>
<th>ULI inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>5</td>
<td>42.4 ± 10.1</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>100</td>
<td>5</td>
<td>2.2 ± 0.2*</td>
<td>94.8</td>
</tr>
<tr>
<td>Friedelin</td>
<td>12.5</td>
<td>4</td>
<td>38.8 ± 11.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Friedelin</td>
<td>25.0</td>
<td>4</td>
<td>35.3 ± 10.8</td>
<td>16.7</td>
</tr>
<tr>
<td>Friedelin</td>
<td>50.0</td>
<td>4</td>
<td>36.5 ± 14.8</td>
<td>13.9</td>
</tr>
</tbody>
</table>

* P < 0.05: Duncan’s test. ANOVA $F_{(4,17)} = 2.84$, $P < 0.05$.

Inhibition of gastric secretion by a water extract from *Baccharis triptera*, Mart. Memórias do Instituto Oswaldo Cruz 86, 137–139.


Short communication

Hepatoprotective effects of bergenin, a major constituent of Mallotus japonicus, on carbon tetrachloride-intoxicated rats

Hwa-Kyung Lim a, Hack-Seang Kim a, Hong-Serck Choi a, Seikwan Oh b, Jongwon Choi c

a Department of Pharmacology, College of Pharmacy, Chungbuk National University, Cheongju, Chungbuk 361-763, South Korea
b College of Medicine, Ewha Womans University, Seoul 158-710, South Korea
c College of Pharmacy, Kyungsung University, Pusan 608-736, South Korea

Received 8 December 1999; received in revised form 20 April 2000; accepted 1 May 2000

Abstract

The hepatoprotective effects of bergenin, a major constituent of Mallotus japonicus, were evaluated against carbon tetrachloride (CCl₄)-induced liver damage in rats. Bergenin at a dose of 50, 100 or 200 mg/kg was administered orally once daily for successive 7 days and then a mixture of 0.5 ml/kg (ip) of CCl₄ in olive oil (1:1) was injected two times each at 12 and 36 h after the final administration of bergenin. The substantially elevated serum enzymatic activities of alanine aspartate aminotransferase, sorbitol dehydrogenase and γ-glutamyltransferase due to CCl₄ treatment were dose dependently restored towards normalization. Meanwhile, the decreased activities of glutathione S-transferase and glutathione reductase were restored towards normalization. In addition, bergenin also significantly prevented the elevation of hepatic malondialdehyde formation and depletion of reduced glutathione content in the liver of CCl₄-intoxicated rats in a dose dependent fashion. The results of this study clearly indicate that bergenin has a potent hepatoprotective action against CCl₄-induced hepatic damage in rats. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Bergenin; Mallotus japonicus; Carbon tetrachloride; Hepatoprotective effect

1. Introduction

Bergenin is a C-glucoside of 4-O-methyl gallic acid that has been isolated from the cortex of Mallotus japonicus (Euphorbiaceae). Malloti Cortex water extract containing about 11–18% bergenin has been used as a folk medicine for treatment and therapy of gastrointestinal diseases such as gastritis, gastric ulcer, diarrhea and constipation (Okada et al., 1973; Abe et al., 1980). In
addition, it has been reported that bergenin has antiinflammatory effects (Swarnalakshmi et al., 1984), antitussive effects (Piegen, 1980) and hypolipidaemic activity (Jahromi et al., 1992). In our laboratory, bergenin was isolated as an active component from Malloti Cortex water extract which was found to have hepatoprotective effects on carbon tetrachloride (CCl4)- and galactosamine-induced hepatotoxicity in rats (Lim et al., 1999). In addition, it was reported that bergenin protected hepatocytes against hepatic damage induced by both CCl4 and galactosamine in primary cultured rat hepatocytes (Kim et al., 2000; Lim et al., 2000). Therefore, to investigate the in vivo hepatoprotective effects, we have assessed the hepatoprotective effects of bergenin using CCl4-intoxicated rats as experimental models.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (150 ± 20 g) were supplied from the Samyuk Laboratory Animal Inc., Osan, Korea. They were housed in polycrylic cages and maintained at 22 ± 1°C and humidity 60 ± 5% with a solid diet and tap water ad libitum. The animals were starved overnight before sacrificed in order to reduce the variation of hepatic metabolism.

2.2. Isolation of bergenin

Cortexes of M. japonicus were collected from Chungbuk Province of Korea and identified by Dr K.S. Lee, Professor, College of Pharmacy, Chungbuk National University. A voucher specimen (CBNU415) was submitted to the herbarium of the University. Dried plant material (1.0 kg) was sliced and extracted three times with 10 l of distilled water for 2 h and filtrate was evaporated under reduced pressure to yield 130 g (13%) of Malloti Cortex water extract. The Malloti Cortex water extract was suspended in methanol. The methanol-soluble portion of the extract was concentrated under reduced pressure and then was carried out using silica gel column with chloroform:methanol (4:1) to give bergenin (19.5 g; 1.95%). Bergenin was purified by several recrystallizations from methanol (Hay and Haynes, 1958) and confirmed by TLC using a ethyl acetate:ethanol:water (100:17:13) as the mobile phase ($R_f = 0.5$) and spots were detected under UV irradiation.

2.3. Bergenin and CCl4 administration

Rats were administered with bergenin 50, 100 and 200 mg/kg orally once a day for 7 days, and then a mixture 0.5 ml/kg (ip) of CCl4 in olive oil (1:1) was injected two times at 12 and 36 h after the final administration of bergenin. Anesthetized in CO2 gas, the rats were decapitated 12 h after the final administration of CCl4.

2.4. Enzyme assay for antihapatotoxic activities

The activities of alanine/aspartate aminotransferase (ALT/AST) and sorbitol dehydrogenase (SDH) were determined by the methods of Reitman and Frankel (1957) and Gerlach (1965), respectively, using an assay kit. γ-Glutamyltransferase (γ-GT) was determined by the methods of Szasz (1969). The contents of glutathione (GSH) and malondialdehyde (MDA) were determined by the methods of Ellman (1959) and Ohkawa et al. (1979), respectively. The activities of glutathione S-transferase (GST) and glutathione reductase (GR) were determined by the method of Habig et al. (1974) and Mize and Langdon (1962), respectively. The protein content was measured by the methods of Lowry et al. (1951) with bovine serum albumin as a standard.

2.5. Statistical analysis

The data were expressed as mean ± SD. The evaluation of statistical significance was determined by Duncan’s new multiple range test. $P$-values < 0.05 were considered statistically significant.
Table 1
Effects of bergenin on serum and liver biochemical indices in CCl₄-intoxicated rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal</th>
<th>CCl₄ control</th>
<th>CCl₄ + bergenin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>50 mg/kg</td>
</tr>
<tr>
<td>ALT (KA unit/ml)</td>
<td>31.6 ± 4.58</td>
<td>90.3 ± 4.97*</td>
<td>76.4 ± 6.38**</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(23.7%)</td>
</tr>
<tr>
<td>AST (KA unit/ml)</td>
<td>54.9 ± 3.82</td>
<td>187.2 ± 16.60*</td>
<td>151.8 ± 9.79**</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(26.8%)</td>
</tr>
<tr>
<td>SDH (U/ml)</td>
<td>19.4 ± 3.44</td>
<td>68.2 ± 7.77*</td>
<td>53.5 ± 6.86**</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(30.1%)</td>
</tr>
<tr>
<td>γ-GT (mU/ml)</td>
<td>23.9 ± 3.60</td>
<td>200.3 ± 20.30*</td>
<td>163.8 ± 18.60**</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(20.7%)</td>
</tr>
<tr>
<td>GSTb</td>
<td>245.6 ± 17.32</td>
<td>138.7 ± 15.64*</td>
<td>186.3 ± 15.78**</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(44.5%)</td>
</tr>
<tr>
<td>GRc</td>
<td>26.8 ± 3.38</td>
<td>12.9 ± 1.62*</td>
<td>17.4 ± 1.29**</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(32.4%)</td>
</tr>
</tbody>
</table>

*Rats were administered with bergenin 50, 100 and 200 mg/kg orally once a day for 7 days, and then CCl₄ 0.5 ml/kg (ip) was injected two times at 12 and 36 h after the final administration of bergenin. The rats were decapitated 12 h after the final administration of CCl₄. Data are expressed as mean ± SD (n = 8). The values in parenthesis are % of protection that is calculated as 100 × (values of CCl₄ control – values of sample)/(values of CCl₄ control – values of normal).

*Units: 1,2-dichloro-4-nitrobenzene nmol/mg protein per min.

**Units: GSH formed nmol/mg protein per min.

*P < 0.01, compared with the normal control.

**P < 0.01, compared with the CCl₄ control by Duncan’s new multiple range test.

3. Results

3.1. Effects of bergenin on AST, ALT, SDH and γ-GT activities

The results of hepatoprotective effect of bergenin on CCl₄-intoxicated rats are shown in Table 1. In the CCl₄-treated control, serum AST, ALT, SDH and γ-GT levels were increased 90.3, 187.2 KA unit/ml, 68.2 sigma unit/ml and 200.3 mU/ml, meanwhile these values showed 31.6, 54.9 KA unit/ml, 19.4 sigma unit/ml and 23.9 mU/ml in normal group, respectively. In contrast, the groups treated with 50, 100 and 200 mg/kg of bergenin decreased significantly the elevated levels of AST, ALT, SDH and γ-GT towards normalization when compared with the CCl₄ control group (P < 0.01).
3.2. Effects of bergenin on hepatic MDA and GSH levels

The production of MDA in CCl₄-treated group was increased by 3.3-fold when compared with the normal group (Fig. 1). Pretreatment with 50, 100 and 200 mg/kg of bergenin reduced CCl₄-induced MDA production in a dose-dependent manner, when compared with the CCl₄ control group \((P < 0.01)\). Administration of CCl₄ decreased the hepatic GSH level by 49%. Pretreatment with 50, 100 and 200 mg/kg of bergenin elevated the hepatic decreased GSH levels towards normalization in a dose-dependent manner (Fig. 2).

3.3. Effects of bergenin on GST and GR activities

GST and GR activities in CCl₄-intoxicated rats were decreased significantly by 43.5 and 51.9%, respectively, when compared with the normal group. Pretreatment with bergenin recovered these decreased enzyme activities produced by CCl₄ towards normalization \((P < 0.01)\) (Table 1).

4. Discussion

Previous in vitro investigations have also shown that bergenin, a major component of \(M. japonicus\), protected hepatocytes against hepatic damage induced by both CCl₄ and galactosamine in primary cultured rat hepatocytes (Kim et al., 2000; Lim et al., 2000). The present study has also demonstrated that bergenin in vivo has a hepatoprotective activity against liver injury induced by CCl₄.

CCl₄ induces hepatotoxicity by metabolic activation, therefore it selectively causes toxicity in liver cells maintaining semi-normal metabolic function. CCl₄ is metabolically activated by the cytochrome \(P_{450}\)-dependent mixed oxidase in the endoplasmic reticulum to form a trichloromethyl free radical \((\cdot \text{CCl}_3)\) which combined with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation (Recknagel et al., 1976; De Groot and Noll, 1986). These result in changes of structures of the endoplasmic reticulum and other membrane, loss of metabolic enzyme activation, reduction of protein synthesis and loss of glucose-6-phosphatase activation, leading to liver damage (Recknagel and Glende, 1973; Gravela et al., 1979; Wolf et al., 1980; Azri et al., 1992).

Hepatotoxic compounds such as CCl₄ are known to cause marked elevation in serum transaminase. In the present study, pretreatment with bergenin attenuated the increases in the activities of AST, ALT, SDH and \(\gamma\)-GT produced by CCl₄ indicating that bergenin protects liver injury induced by CCl₄ towards normalization.

It has been hypothesized that one of the principal causes of CCl₄-induced liver injury is lipid peroxidation by free radical derivatives of CCl₄. In states of oxidative stress, GSH is converted to GSSG and depleted leading to lipid peroxidation. Therefore, the role of GSH as a reasonable maker for the evaluation of oxidative stress is important (Recknagel et al., 1991). Bergenin inhibited significantly lipid peroxidation and recovered the decreased hepatic GSH level induced by CCl₄ towards normalization.

To prevent lipid peroxidation, it is very important to maintain the level of GSH. GSSG is...
reduced to GSH by GR, which is NADPH-dependent. It plays a role in maintaining adequate amounts of GSH. Accordingly, the reduction of GR results in decreasing GSH (Recknagel et al., 1991). In CCl₄-intoxicated rats, the activity of GR is markedly decreased. However, bergenin preserved the activity of GR up to 57.3% at 200 mg/kg of that of CCl₄ control group.

GST is a soluble protein located in cytosol which plays an important role in the detoxification and excretion of xenobiotics (Boyer et al., 1984; Masukawa and Iwata, 1986). The function of GST is divided into catalysis and binding. GST catalyzes the conjugation of the thiol functional groups of glutathione to electrophilic xenobiotics and results in increasing solubility. The xenobiotic-GSH conjugate is then either eliminated or converted to mercapturic acid. Another function of GST is the binding between GSH and endogenous or exogenous substances. Since GST increases solubility of hydrophobic substances, it plays an important role in storage and excretion of xenobiotics. Compounds which increase the activity of GST, which metabolizes toxic compounds to non-toxic ones, means they have an increasing protective activity of the liver. In CCl₄-intoxicated rats, the activity of GST is decreased 43.5% compared to that of normal group. The activity of GST recovered returning up to 48.1% at 200 mg/kg of bergenin compared to that of CCl₄ control group.

From the above results, we concluded that the hepatoprotective activity of bergenin against CCl₄-intoxicated rats. Also, the activity of GST related to detoxification was increased towards normalization. In addition, the activity of GR, suppressing free radical and the content of GSH reduced by free radical were affected by bergenin. Therefore it is assumed that effects of bergenin on liver protection are related by glutathione-mediated detoxification as well as free radical suppressing activity. In addition, it is demonstrated that the present in vivo results such as normalization by bergenin of elevated ALT and SDH or decreased GR, GST activities and GSH content are consistent with those of previous in vitro study.

**Acknowledgements**

The present study was supported by the grant of Research Foundation, Chungbuk National University (1999–2000).

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Lim, H.K., Kim, H.S., Chung, M.W., Kim, Y.C., 2000. Protective effects of bergenin, the major constituent of *Mallo-
Short communication

Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses

Seong-Kug Eo, Young-So Kim, Chong-Kil Lee, Seong-Sun Han *

*College of Pharmacy, Chungbuk National University, Cheongju 361-763, South Korea*

Received 30 June 1999; received in revised form 1 May 2000; accepted 10 May 2000

Abstract

Two protein bound polysaccharides, a neutral protein bound polysaccharide (NPBP) and an acidic protein bound polysaccharide (APBP), were isolated from water soluble substances of *Ganoderma lucidum* by EtOH precipitation and DEAE–cellulose column chromatography. Their antiviral activities against herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) were then investigated by plaque reduction assay. APBP exhibited more potent HSV-1 and HSV-2 antiviral activity than NPBP with 50% effective concentration (EC₅₀) of 300–520 µg/ml. In order to examine the possible mode of the antiviral activity of APBP its virucidal effect, antiviral activity in preincubation, attachment and penetration assay were tested with HSV-1 and HSV-2. APBP was found to have a direct virucidal effect on HSV-1 and HSV-2. APBP did not induce IFN or IFN-like materials in vitro and is not expected to induce a change from a normal state to an antiviral state. APBP in concentrations of 100 and 90 µg/ml inhibited up to 50% of the attachment of HSV-1 and HSV-2 to Vero cells and was also found to prevent penetration of both types of HSV into Vero cells. These results show that the antipheretic activity of APBP seems to be related to its binding with HSV-specific glycoproteins responsible for the attachment and penetration, and APBP impedes the complex interactions of viruses with cell plasma membranes. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Ganoderma lucidum*; Acidic protein bound polysaccharide; Antiviral activity; Herpes simplex viruses; Attachment; Penetration

1. Introduction

*Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae) in basidiomycetous fungi has been used to treat various human diseases such as hepatopathy, chronic hepatitis, nephritis, gastric ulcer, hy-
pertension and tumorigenic diseases in oriental folk medicine (Kim and Kim, 1990).

*G. lucidum* was reported to have many biologically active components (Lee and Rhee, 1990; Kawagishi et al., 1993; Lin et al., 1995). Especially, the biological activity of polysaccharides and protein bound polysaccharides has been studied on antitumor activity (Mizuno et al., 1985). Several β-glucans, appertaining to the same molecular species but having different degrees of branching, were isolated from water and alkali extracts (Miyazaki and Nishijima, 1982; Mizuno et al., 1985). Most of the antitumor β-glucans were reported to contain a branched glucan core with (1→3)-β-, (1→4)-β-, and (1→6)-β-linkages and an average molecular weight of 1 050 000 Da (Mizuno et al., 1984). Their antitumor activity was reported to be accomplished by enhancing the host mediated mechanisms including increased IL-2 production (Lei and Lin, 1992) and the stimulation of cytotoxic T lymphocytes, NK activity (Won et al., 1989) and antibody production (Kim et al., 1993).

Sulfated polysaccharides such as carrageenan, xylofuranan sulfate, ribofuranan sulfate and dextran sulfate have been attractive candidates for possible antiviral drugs because of their potent in vitro activities against human immunodeficiency virus (HIV), herpes simplex virus (HSV) and other enveloped viruses (Nakashima et al., 1987; Chang et al., 1988). The activities of these sulfated polysaccharides are linked to the anionic features of the molecules (Marchetti et al., 1994). The mode of antiviral action of these polysaccharides was suggested to be attributed to an inhibiton of virus binding to the cells, inhibition of virus-cell fusion, or inhibition of both virus-cell binding and fusion (Hosoya et al., 1991).

Also, we knew that acidic protein bound polysaccarhides (APBP) isolated from the carpophores of *G. lucidum* had shown more potent antiherpetic activity than neutral protein bound polysaccharides (NPBP) in this study. This discovery induced further detailed analysis of the antiviral properties of APBP on HSV. Therefore, its virucidal effect, antiviral activity in preincubation, attachment and penetration assay were tested with HSV-1 and HSV-2 in order to examine its possible mode of antiviral activity.

### 2. Materials and methods

#### 2.1. Materials and reagents

Artificially grown carpophores of *Ganoderma lucidum* (Fr.) Karst. (Ganodermataceae) were purchased from a local herbal drug store and authenticated by Dr Byung Kak Kim, Department of Microbial Chemistry, Seoul National University. A voucher specimen (No. Cpm 605) has been deposited at the Medicinal Plants Herbarium of Chungbuk National University. Eagle's minimum essential medium (MEM), trypsin, penicillin, streptomycin and amphotericin B were purchased from Gibco BRL (Gaithersburg, MD). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet were purchased from Sigma (St. Louise, MO). Vero cell (African green monkey kidney cell, ATCC CCL 81), HEp-2 cell (human epidermoid carcinoma cell of larynx, ATCC CCL 23), herpes simplex virus type 1 (HSV-1) F strain ATCC VR-733 and type 2 (HSV-2) G strain ATCC VR-734 were obtained from the American Type Culture Collection (Rockville, MD).

#### 2.2. Isolation of NPBP and APBP

The carpophores of *G. lucidum* (500 g) were extracted with hot water for 8 h. The water extract was concentrated to one tenth of the original volume, and added to three volumes of ice cold EtOH to precipitate the high molecular weight components (GLhw). Then the sample was allowed to stand overnight at 4°C. It was centrifuged and the precipitate obtained was lyophilized. The lyophilized GLhw (3.88 g) was a brownish powder with high molecular weight components of water soluble substances. The GLhw was separated into neutral and acidic fractions by using DEAE–cellulose column chromatography. The GLhw (3 g) was applied onto the DEAE–cellulose (Cl− form, Sigma, USA) column (bed volume = 50 ml). It was eluted with H2O (pH = 7.2) followed by 2 M NaCl. The anthrone (620 nm) and Lowry–Folin tests (540 nm) were performed against each fraction. The fraction shown by the positive anthrone and
Lowry–Folin tests was designated as the neutral protein bound polysaccharide (NPBP, white powder, 0.55 g). The other brownish and acidic fraction which showed positive anthrone and Lowry–Folin tests was designated as the acidic protein bound polysaccharide (APBP, brownish powder, 0.85 g).

2.3. Cells and viruses

Vero and HEp-2 cells were cultured with MEM supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 I.U./ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were subcultured twice a week. HSV-1 and HSV-2 stocks were prepared in Vero cell cultures and stored at −70°C until use. Viral titer was determined using plaque assay.

2.4. Cytotoxicity assay

For cytotoxicity assay Vero cells were seeded in 96 well plates (Falcon, NJ, USA) at an initial density of 3.5 × 10^4 cells per well. After incubation of the cells for 16–18 h at 37°C various concentrations of APBP were added, and the incubation was continued for 48 h. Viable cell yield was determined by MTT reduction assay according to a reported procedure (Scubiero et al., 1988). The cytotoxicity was expressed as the 50% cytotoxic concentration (CC₅₀) which is the concentration of substances needed to inhibit the growth of cells up to 50% by regression analysis.

2.5. Plaque reduction assay

The antiviral activity was essentially evaluated by a plaque reduction assay (Shigeta et al., 1992). Host cell monolayers grown in 24 well culture plates (Falcon, NJ, USA) were infected with about 150 pfu of virus per well in the absence or presence of various concentrations of NPBP or APBP. After 1 h adsorption agar overlay medium containing substances at various concentrations was overlaid. After 1–3 days of incubation at 37°C, the virus plaques were counted. The degree of inhibition was expressed as the 50% effective concentration (EC₅₀) which was calculated as the concentration of substances required to reduce virus plaque up to 50% by regression analysis. Antiviral activity for each substance was evaluated by selectivity index (SI) which was calculated by dividing the CC₅₀ by the EC₅₀.

2.6. Virucidal effect

Assays were performed according to the method previously described (Barnard et al., 1992). Virus stock solution (> 10⁶ pfu/ml) was mixed with APBP of 0.5, 1.0 and 1.5 × 10³ μg/ml in equal volumes, and incubated at 5% CO₂, 37°C for 1, 2 and 3 h. Surviving virus titers were determined by the plaque reduction assay in Vero cells.

2.7. Antiviral activity in preincubation

In order to determine the effect on the antiviral activity of APBP when incubated with Vero cells prior to infection with the virus the procedure was carried out according to the previously published method (Katz et al., 1991) with some modification. Vero cells were preincubated with APBP of 0.5, 1.0 and 1.5 × 10³ μg/ml or IFN-α (Boehringer Mannheim, Mannheim, Germany) of 200 and 400 I.U./ml at 5% CO₂, 37°C for 1, 2 and 24 h, respectively. After rinsing the cells with PBS (pH 7.4), the cells were infected with virus, and then incubated at 5% CO₂, 37°C until plaques formed. The virus plaques were counted.

2.8. Attachment assay

The effect on attachment of HSV-1 and HSV-2 to Vero cells in the presence of APBP was tested as previously described (Barnard et al., 1993). Virus was diluted in serum free MEM to 100 CCID₅₀/ml and attached to Vero cells in 12 well culture plates at about 80% confluence. Attachment was for various time periods up to 3 h at 4°C in the absence and presence of APBP. At the appropriate time point unattached virus was removed and the cells overlayed with agar overlay medium and incubated at 37°C until plaques
formed. The virus plaques were then counted. To determine an EC\textsubscript{50} of the inhibition of viral attachment virus was allowed to attach for 3 h in the absence and presence of APBP at various concentrations of 0.01, 0.1, 0.2, 0.5, 1.0 and 1.5 \times 10^3 \mu g/ml. The EC\textsubscript{50} value was calculated by regression analysis of the dose–response curve generated from the data.

2.9. Penetration assay

The assay was done as previously described (Rosenthal et al., 1985). The virus was diluted in serum free MEM to 100 CCID\textsubscript{50}/ml and attached to Vero cells in a 12 well culture plate at about 80% confluence. Attachment was for 3 h at 4°C. After 3.5 h the temperature was abruptly increased to 37°C to maximize penetration of the virus. Penetration was proceeded for various time periods up to 60 min after the increase of temperature in the absence and presence of an equal volume of APBP. Any remaining attached virus was neutralized by the addition of PBS (pH 3.0) for 1 min. After several washes with PBS (pH 7.4) the cells were overlayed with agar overlay medium to quantify surviving virus versus time of drug exposure. To determine an EC\textsubscript{50} of inhibition of virus penetration, the virus was allowed to penetrate for 60 min as described above in the absence and presence of APBP at various concentrations of 0.01, 0.1, 0.2, 0.5, 1.0 and 1.5 \times 10^3 \mu g/ml. The EC\textsubscript{50} value was calculated by regression analysis of the dose response curve generated from the data.

2.10. Statistical analysis

The data were expressed as mean ± S.D. The statistical significance of the difference between mean values was determined by Student’s t-test. Data were considered different at a significance level of \( P < 0.05 \).

3. Results and discussion

To investigate the antiviral activities of protein bound polysaccharides from \textit{G. lucidum} two protein bound polysaccharides, NPBP and APBP were isolated from water soluble substances of the carpophores by EtOH precipitation and DEAE–cellulose column chromatography. Their antiviral activities against HSV-1 and HSV-2 were examined by a plaque reduction assay. NPBP and APBP exhibited potent antiviral activity as described in Table 1. APBP inhibited plaque formations of both types of HSV more potently than NPBP with an EC\textsubscript{50} of 300–520 \mu g/ml. APBP was identified as consisting mainly of polysaccharide (approximately 40.6\%) and protein (approximately 7.8\%) by the anthrone test and the Lowry–Folin test. APBP showed the usual molar ratio (C:H:O = 1:2:1) of carbohydrates by elemental analysis. Most of the antiviral or antitumor polysaccharides isolated from the hot water extract of \textit{G. lucidum} were reported to be a branched \( \beta \)-glucans in (1→3)-\( \beta \)-, (1→4)-\( \beta \)- and (1→6)-\( \beta \)-linkages (Mizuno et al., 1984). This suggests that these \( \beta \)-glucans may be the protein.

Table 1

Antiviral activities of NPBP and APBP on herpes simplex viruses by plaque reduction assay

<table>
<thead>
<tr>
<th>Polysaccharides</th>
<th>Host cells</th>
<th>CC\textsubscript{50} (\times 10^3 \mu g/ml)</th>
<th>EC\textsubscript{50} (\times 10^3 \mu g/ml)</th>
<th>SI\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HSV-1\textsuperscript{b}</td>
<td>HSV-2\textsuperscript{c}</td>
<td>HSV-1</td>
</tr>
<tr>
<td>NPBP</td>
<td>Vero</td>
<td>&gt;10.0</td>
<td>1.29</td>
<td>&gt;7.75</td>
</tr>
<tr>
<td></td>
<td>Hep-2</td>
<td>&gt;10.0</td>
<td>0.45</td>
<td>&gt;22.22</td>
</tr>
<tr>
<td>APBP</td>
<td>Vero</td>
<td>&gt;10.0</td>
<td>0.30</td>
<td>&gt;33.33</td>
</tr>
<tr>
<td></td>
<td>Hep-2</td>
<td>&gt;10.0</td>
<td>0.33</td>
<td>&gt;30.30</td>
</tr>
</tbody>
</table>

\( ^a \) SI = CC\textsubscript{50}/EC\textsubscript{50}.

\( ^b \) HSV-1, herpes simplex virus type 1 F strain.

\( ^c \) HSV-2, herpes simplex virus type 2 G strain.
bound polysaccharides that exhibit antiherpetic activity. It appears that the protein and polysaccharide were bound together since the protein moiety was not completely removed during the purification. However, the entity of binding is uncertain. Also, these results suggest that the antiviral activities of protein bound polysaccharide is related to the net of electric charge.

It is known that the antiviral activities of polysaccharides are linked to the anionic features of the molecules and that they inhibit the early stages of viral infection such as attachment and penetration (Marchetti et al., 1994). Therefore, APBP was tested for its virucidal effect, antiviral activity in preincubation, and its effect on the attachment and penetration assay of HSV-1 and HSV-2 in order to examine its possible mode of antiviral activity. As for its virucidal effect, APBP was found to significantly decrease the surviving viral titer in a dose dependent manner, as compared with each VC group. This virucidal effect is assumed to be irreversible (Fig. 1). As for its antiviral activity. It was found to significantly decrease the surviving viral titer in a dose dependent manner, as compared with each VC group. This antiviral effect is assumed to be irreversible. *G. tsugae* mycelium has been reported to enhance splenic natural killer cell activity and serum IFN production in mice (Won et al., 1992). Accordingly, in order to testify whether APBP also enhance the induction of IFN or IFN-like materials in vitro the effect of preincubation of Vero cells with APBP for various time intervals and the subsequent infectivity of HSV-1 and HSV-2 was examined. The number of plaques formed by both types of HSV in Vero cells which had been preincubated with APBP was not significantly different from the VC group as shown in Fig. 2. These results differed from the positive control group. For the positive control group Vero cells were preincubated with 200 and 400 I.U./mL of IFN-α for 24 h and the IFN-α was removed with PBS (pH 7.4) before addition of virus. For this group, the number of plaques per well was abruptly decreased. These results suggest that APBP may not induce IFN or IFN-like materials in vitro and is not expected to induce a change of Vero cells from a normal state to an antiviral state.

Viruses were attached to cells at 4°C in the presence of APBP for various time periods up to 3 h. The assay was terminated by removing the virus and APBP with PBS (pH 7.4). The attachment of both viruses was dramatically inhibited by treatment with APBP. This inhibitory effect on attachment was evaluated as described in Table 2. Viruses were allowed to attach for 3 h in the absence and presence of APBP at various concentrations of 0.01, 0.1, 0.2, 0.5, 1.0 and 1.5 × 10^3 μg/mL. The EC_{50} value was then calculated by regression analysis of the dose response curve generated from the data. APBP was found to inhibit up to 50% of the attachment of HSV-1 and HSV-2 at concentrations of 100 and 90 μg/mL, respectively. Also, to examine the effect of APBP on penetration viruses were allowed to attach at 4°C for 3 h. This temperature is sufficient for attachment but discourages penetration. The temperature was abruptly raised up to 37°C to facilitate penetration, either in the absence and
Inhibitory effect of APBP on the plaque formation of HSV-1 and HSV-2 requires prolonged exposure to culture of Vero cells throughout. Vero cells were preincubated with APBP (0.5, 1.0 and 1.5 $10^3$ μg/ml) for 1, 2 and 24 h, and IFN-α (200 and 400 I.U./ml) for 24 h as positive control group, respectively. APBP and IFN-α were washed twice with PBS (pH 7.4) immediately prior to inoculation with virus. The sharp indicates that the value is significantly different from each virus control (VC) group (***P < 0.001; **P < 0.01).

Extracellular viruses were neutralized with PBS (pH 3.0) after incubation for various time periods. APBP appeared to significantly prevent penetration of both types of HSV after prolonged exposure of 60 min. APBP was identified as inhibiting penetration of HSV-1 and HSV-2 into Vero cells with an EC$_{50}$ of 0.83 and 1.40 $10^3$ μg/ml, respectively (Table 2). Therefore, the antiviral activity of APBP may be due mainly to the inhibition of the attachment of HSV to Vero cells, and also the inhibitory effect on penetration was assumed to assist in increasing the antiviral activity of APBP. These inhibitory effects of APBP on the attachment and penetration is suggested to be due to selective binding of HSV-specific glycoproteins responsible for attachment and penetration such as gB, gC and gD (Roizman and Amy, 1993) and subsequently preventing the function of these glycoproteins in the process of attachment and penetration.

In conclusion, the antiviral activity of APBP may be mainly due to the inhibition of the attachment of HSV to Vero cells, and also the inhibitory effect on penetration is assumed to augment the antiviral activity of APBP.

Acknowledgements

This work was supported by a grant from the Regional Center for Health and Life Science,

Table 2
Inhibitory effect of APBP on attachment and penetration of HSV-1 and HSV-2 to Vero cells

<table>
<thead>
<tr>
<th>Viruses</th>
<th>Attachment (×10³ μg/ml)</th>
<th>Penetration (×10³ μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC$_{50}$</td>
<td>EC$_{70}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC$_{50}$</td>
</tr>
<tr>
<td>HSV-1</td>
<td>0.10</td>
<td>0.16</td>
</tr>
<tr>
<td>HSV-2</td>
<td>0.09</td>
<td>0.15</td>
</tr>
</tbody>
</table>

a To determine an EC$_{50}$ of inhibition of virus attachment, viruses were allowed to attach for 3 h in the absence or presence of APBP at 0.01, 0.1, 0.2, 0.5, 1.0 and 1.5 $10^3$ μg/ml and the EC$_{50}$ value was calculated by regression analysis of the dose–response curve generated from the data.

b To determine an EC$_{50}$ of inhibition of virus penetration, attached viruses on cells were allowed to penetrate for 60 min in absence or presence of APBP at 0.01, 0.1, 0.2, 0.5, 1.0 and 1.5 $10^3$ μg/ml and the EC$_{50}$ value was calculated by regression analysis of the dose–response curve generated from the data.
Chungbuk National University. We sincerely thank Amanda Royer and Chuck Gouffon for their reviews and comments on the manuscript.

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Short communication

Phytochemical flavones isolated from *Scutellaria barbata* and antibacterial activity against methicillin-resistant *Staphylococcus aureus*

Yoichi Sato a,b, Shiho Suzaki a, Takako Nishikawa a, Masaru Kihara a, Hirofumi Shibata a, Tomihiko Higuti a,*

a Laboratory of Microbial Chemistry, Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi 1-78, Tokushima 770-8505, Japan
b Alps Pharmaceutical Industries, Co., Ltd., 10-50, 2-Chome, Mukaimachi, Furukawa, Gifu 509-4241, Japan

Received 30 November 1999; received in revised form 13 April 2000; accepted 10 May 2000

Abstract

A crude extract prepared from *Scutellaria barbata* D. Don (Lamiaceae) was analyzed in the effort to discover antibacterial compounds against high-level strains of methicillin-resistant *Staphylococcus aureus* (MRSA). Apigenin and luteolin were isolated from the plant as active constituents against the bacteria. These flavonoid congeners were selectively toxic to *S. aureus*, including the MRSA and methicillin-sensitive *S. aureus* strains. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: *Scutellaria barbata*; Antibacterial activity; Apigenin; Luteolin; Methicillin-resistant *Staphylococcus aureus*

1. Introduction

*Scutellaria barbata* D. Don (Lamiaceae) is a perennial herb which is natively distributed throughout southern China. This herb is known in traditional Chinese Medicine as Ban-Zhi-Lian and has been used as an anti-inflammatory and antitumor agent and as a diuretic (Jiangsu New Medical College, 1977). The herb is known to contain alkaloids and flavones (Jiangsu New Medical College, 1977).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is responsible for worldwide outbreaks of nosocomial infections (Voss and Doebbeling, 1995). According to recent studies, the isolation of MRSA is no longer limited to patients at risk for nosocomial infection or with other predisposing factors; community-acquired or outpatient MRSA infections are increasing in both children
and adults (Moreno et al., 1995; Herold et al., 1998). At present, however, the pharmaceutical arsenal available to control MRSA is limited.

During the course of our screening of plant medicinal resources for antibacterial agents against MRSA (Sato et al., 1997a,b), a crude extract prepared from *S. barbata* was identified as a candidate for further isolation studies. In this paper, we report isolation of active principles from the extract and their antibacterial properties.

2. Materials and methods

2.1. Plant material

Dried plant materials of *S. barbata* were obtained from Tochimototenkai-Do Co., Ltd. (Osaka, Japan). Identification of the plant material was confirmed by Dr K. Murakami, and a voucher specimen (No. 98S-525-015) is stored in the Herbarium of University of Tokushima, Faculty of Pharmaceutical Sciences (Tokushima, Japan).

2.2. Extraction and isolation

Target substances were extracted from aerial parts of *S. barbata*. The extracts were analyzed by thin-layer chromatography (TLC) on precoated silica gel 60 F$_{254}$ plates (Merck, Darmstadt, Germany) with chloroform-methanol, 9:1 (v:v) as the mobile phase. The material was coarsely ground before extraction. A total of 1.5 kg of the material was extracted three times with 50% ethanol (45 l) under reflux for 2 h. The combined extracts were evaporated and partitioned with diethyl ether (9 l) and butanol saturated with water (45 l). Only the fraction extracted in diethyl ether showed remarkable anti-MRSA activity. This fraction (214.1 g) was chromatographed on a 2.5 × 30-cm silica gel 60 column (Merck) eluted stepwise with n-hexane, n-hexane-ethyl acetate, 1:1, and finally ethyl acetate. Anti-MRSA activity was detected in the fractions eluted with n-hexane-ethylacetate (1:1) and with ethyl acetate. These fractions were further separated on a 4 × 7.5-cm ODS-Q3 (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) column eluted with methanol (50, 80 and 100%). Anti-MRSA activity was detected in the 50%-methanol eluates (the eluate from the n-hexane-ethylacetate (1:1) fraction was designated as ‘fraction 1’ and that from the ethyl acetate-fraction was designated as ‘fraction 2’). Finally, fraction 1 was purified by high performance liquid chromatography (HPLC) on LiChrosorb Si 60 (2.5 × 25 cm; Merck) with n-hexane-ethyl acetate, 1:9, to afford SB-1 (920 mg); fraction 2 was purified by the HPLC with chloroform-methanol, 9:1, to afford SB-2 (25 mg). Purity was monitored by TLC and by HPLC on LiChrosorb Si 60 (4.0 mm × 25 cm; Merck) with n-hexane-ethyl acetate, 1:1, as the mobile phase. Detection was performed at 254 nm.

2.3. Preparation of bacterial cells

MRSA and methicillin-sensitive *S. aureus* (MSSA) strains listed in Table 1 and other bacterial strains listed in Table 3 were from laboratory stock cultures. The MRSA strains were defined on the basis of the occurrence of the *mecA* gene and of their resistance to methicillin and oxacillin, according to the guidelines of the National Committee for Clinical Laboratory Standards (1997). After culturing all strains on Mueller–Hinton agar (Difco, Detroit, MI), the cells were resuspended in Mueller–Hinton broth (Difco) to give 10$^8$ colony-forming units/ml; the resuspended cells were then incubated.

2.4. Detection of *mecA* gene

Detection of the *mecA* gene in strains of MRSA and MSSA was performed by PCR amplification. Template DNA was obtained from 0.5 ml of broth cultured overnight (ca. 10$^8$ colony-forming units/ml) after lysis in achromopeptidase (50 000 U/ml; Wako Pure Chemicals Industries, Ltd.), as previously described (Hiramatsu et al., 1992).

The PCR assay was performed in a DNA thermal cycler, a Program Temp Control System PC-700 (Astec Co. Ltd., Shimen, Japan), by using a Gene Taq Amplifying Kit (Wako Pure Chemicals Industries, Ltd.), according to the manufacturer’s
recommendations. Synthetic oligonucleotides used as primers were 5’-GTAGAAATGACTGAACGTCCGATAA-3’ and 5’-CCAATTCACATTGTTTCGTTCTAA-3’ (Hokkaido System Science Co. Ltd., Sapporo, Japan); these primers correspond to nucleotides from positions 318–342 and 603–627, respectively, of the mecA gene sequence (Geha et al., 1994).

2.5. Determination of antibacterial activity

During the extraction and purification procedure, disc-diffusion tests were performed to examine antibacterial activity with Whatman AA discs (13.0 mm; Whatman International Ltd, Maidstone, UK) containing various concentrations of extract. The discs were placed on Mueller–Hinton agar inoculated with 10^5 colony-forming units/ml of MRSA. The zone of inhibition was determined after incubation at 37°C for 20 h. To estimate antibacterial activity of purified isolates, a minimum inhibitory concentration (MIC) was determined using Mueller–Hinton agar according to the method described by the Japanese Society for Antimicrobial Chemotherapy (1981). Cell suspensions (1 × 10^6 colony-forming units/ml) of the examined bacteria were inoculated onto agar plates using a replicating device. Plates were read after 20 h incubation at 37°C.

3. Results and discussion

The 50% ethanolic extract of dried aerial parts of S. barbata was partitioned with diethyl ether, butanol, and water. Among them, only the diethyl ether extract showed notable antibacterial activity against the MRSA strains. A combination of silica gel (Wako gel C-300, Kiesel gel 60, and LiChrosorb Si60) and reversed-phase (ODS-Q3) column chromatographies of the diethyl ether extract afforded anti-MRSA active compounds, which were referred to as SB-1 and SB-2 (yield from the plant, 0.06 and 0.0017%, respectively).

Table 1
Correlation of carriage of the mecA gene and MICs of 20 S. aureus strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>mecA*</th>
<th>MIC (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Methicillin</td>
</tr>
<tr>
<td>1.</td>
<td>+</td>
<td>500</td>
</tr>
<tr>
<td>2.</td>
<td>+</td>
<td>500</td>
</tr>
<tr>
<td>3.</td>
<td>+</td>
<td>500</td>
</tr>
<tr>
<td>4.</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>5.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>6.</td>
<td>+</td>
<td>250</td>
</tr>
<tr>
<td>7.</td>
<td>+</td>
<td>250</td>
</tr>
<tr>
<td>8.</td>
<td>+</td>
<td>500</td>
</tr>
<tr>
<td>9.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>10.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>11.</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>12.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>13.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>14.</td>
<td>+</td>
<td>1000</td>
</tr>
<tr>
<td>15.</td>
<td>+</td>
<td>15.6</td>
</tr>
<tr>
<td>16.</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>17.</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>18.</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>19.</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>20.</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

*a*, mecA positive; *, mecA negative.
Table 2
MICs of the ether extract, the isolate SB-1, apigenin, and luteolin against 20 *S. aureus* strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC (µg/ml)</th>
<th>Ether ext.</th>
<th>SB-1</th>
<th>Apigenin</th>
<th>Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0001</td>
<td>250</td>
<td>15.6</td>
<td>7.8</td>
<td>125</td>
</tr>
<tr>
<td>2.</td>
<td>0002</td>
<td>125</td>
<td>7.8</td>
<td>3.9</td>
<td>125</td>
</tr>
<tr>
<td>3.</td>
<td>0003</td>
<td>250</td>
<td>15.6</td>
<td>7.8</td>
<td>125</td>
</tr>
<tr>
<td>4.</td>
<td>0004</td>
<td>250</td>
<td>15.6</td>
<td>7.8</td>
<td>125</td>
</tr>
<tr>
<td>5.</td>
<td>0005</td>
<td>125</td>
<td>7.8</td>
<td>3.9</td>
<td>62.5</td>
</tr>
<tr>
<td>6.</td>
<td>0006</td>
<td>125</td>
<td>7.8</td>
<td>3.9</td>
<td>62.5</td>
</tr>
<tr>
<td>7.</td>
<td>0007</td>
<td>125</td>
<td>3.9</td>
<td>3.9</td>
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<td>8.</td>
<td>0008</td>
<td>125</td>
<td>7.8</td>
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<td>62.5</td>
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<tr>
<td>9.</td>
<td>0009</td>
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<td>15.6</td>
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</tr>
<tr>
<td>10.</td>
<td>0010</td>
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<td>15.6</td>
<td>15.6</td>
<td>62.5</td>
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<tr>
<td>11.</td>
<td>0016</td>
<td>250</td>
<td>31.3</td>
<td>7.8</td>
<td>62.5</td>
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<tr>
<td>12.</td>
<td>0017</td>
<td>&gt;250</td>
<td>31.3</td>
<td>7.8</td>
<td>125</td>
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<tr>
<td>13.</td>
<td>0018</td>
<td>250</td>
<td>15.6</td>
<td>7.8</td>
<td>125</td>
</tr>
<tr>
<td>14.</td>
<td>0019</td>
<td>250</td>
<td>15.6</td>
<td>7.8</td>
<td>125</td>
</tr>
<tr>
<td>15.</td>
<td>0020</td>
<td>125</td>
<td>7.8</td>
<td>3.9</td>
<td>125</td>
</tr>
<tr>
<td>16.</td>
<td>1020</td>
<td>&gt;250</td>
<td>15.6</td>
<td>15.6</td>
<td>62.5</td>
</tr>
<tr>
<td>17.</td>
<td>1023</td>
<td>250</td>
<td>31.3</td>
<td>3.9</td>
<td>62.5</td>
</tr>
<tr>
<td>18.</td>
<td>1029</td>
<td>250</td>
<td>7.8</td>
<td>3.9</td>
<td>125</td>
</tr>
<tr>
<td>19.</td>
<td>1032</td>
<td>250</td>
<td>15.6</td>
<td>15.6</td>
<td>125</td>
</tr>
<tr>
<td>20.</td>
<td>1100</td>
<td>250</td>
<td>31.3</td>
<td>15.6</td>
<td>62.5</td>
</tr>
</tbody>
</table>

SB-1 was obtained as a yellow needle with an mp of over 300°C. The compound showed a molecular ion peak at m/z 270 in the electron ionization (EI)-mass spectrum. The molecular formula of SB-1 was determined by high-resolution mass spectrometry to be C_{15}H_{10}O_{5}. SB-2 was obtained as a yellow needle with an mp of over 300°C. The compound showed a molecular ion peak at m/z 286 in the EI-mass spectrum, and its molecular formula was determined by high-resolution mass spectrometry to be C_{15}H_{10}O_{6}. By comparing the *R*<sub>f</sub> values from the TLC, as well as the infrared (IR) and nuclear magnetic resonance (NMR) spectra with those of authentic samples, these compounds were identified as flavone derivatives. SB-1 was identified as apigenin and SB-2 was identified as luteolin.

Table 1 shows MICs of methicillin, oxacillin, and vancomycin against *S. aureus*. Of 20 strains used in the present study, 15 strains were *mecA*-positive and five strains *mecA*-negative. With the exception of strain 0020, which was susceptible to oxacillin, all of the *mecA*-positive strains showed MICs equal to or greater than 250 µg/ml, indicating that these strains were highly resistant MRSA. The *mecA*-negative strains were susceptible to the antibiotics, indicating that these strains were of the MSSA type. In contrast, no strains were resistant to vancomycin.

MICs of the Et<sub>2</sub>O-extract, the isolate SB-1, and the authentic compounds apigenin and luteolin against *S. aureus* are summarized in Table 2. Because of the small yield of the SB-2 isolate, its MIC remains to be examined. In comparison with the Et<sub>2</sub>O-extract, the antibacterial potency of SB-1 was remarkably improved to levels nearly the same as those achieved by apigenin. The authentic compound of SB-2, luteolin, also showed antibacterial activity that differed from activity reported in earlier studies (Miski et al., 1983; Mori et al., 1987). Nonetheless, luteolin was not as potent as apigenin. No differences in susceptibility to the two compounds were detected in the MRSA and MSSA strains (MIC, 3.9–15.6 µg/ml for apigenin and 62.5–125 µg/ml for luteolin). These findings indicate that these compounds were uniformly active against all strains of MRSA and MSSA.
In general, Gram-negative bacteria are less sensitive to the inhibitory action of phytoalexin than are Gram-positive bacteria (Kurosaki and Nishi, 1983). Furthermore, previous studies (Miski et al., 1983; Osawa et al., 1992) have demonstrated that apigenin and luteolin exhibited no antibacterial activity against some Gram-positive coccal strains, including *Staphylococcus epidermidis*. Therefore, we examined the antibacterial activity of the two compounds against other bacteria (Table 3). According to our expectations, the two compounds exhibited no appreciable antibacterial potency against the bacteria. Our findings indicate that these compounds were selectively toxic to *S. aureus*.

The antibacterial compounds discussed here belong to the flavones, a class of flavonoids. These naturally occurring compounds are ubiquitous in vascular plants and possess a wide variety of cellular and biochemical effects (Havsteen, 1983; Pathak et al., 1991; Brandi, 1992). Many reports have discussed their antibacterial effects (Miski et al., 1983; El-Gammal and Mansour, 1986; Mori et al., 1987; Nishino et al., 1987; Inuma et al., 1994; Tsuchiya et al., 1994; Sato et al., 1996; Tsuchiya et al., 1996). The flavonoids have not been associated with serious toxicity (Fullas et al., 1994) and have already been used in medicine (Havsteen, 1983; Pathak et al., 1991; Brandi, 1992). In the present study, apigenin and luteolin are characterized as selectively inhibiting the growth of *S. aureus*, including MRSA. Our findings suggest that apigenin and the related flavonoids are potentially useful for the development of therapeutic treatments of MRSA infections.

**Acknowledgements**

The authors thank Dr K. Murakami for identification of the plant material. This work was partly supported by a Grant-in-Aid for Scientific Research, No. 07558095, from the Ministry of Education, Science and Culture of Japan.

**References**


**Table 3**

**Antibacterial properties of apigenin and luteolin**

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/ml)</th>
<th>Apigenin</th>
<th>Luteolin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus epidermidis</em> IFO 3762</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em> IFO 3514</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> ATCC 21212</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em> ATCC 19606</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Citrobacter freundii</em> ATCC 8090</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em> IFO 13535</td>
<td>&gt;250</td>
<td>&gt;250</td>
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<tr>
<td><em>Escherichia coli</em> NIHJ JC-2</td>
<td>&gt;250</td>
<td>&gt;250</td>
<td></td>
</tr>
<tr>
<td><em>Proteus mirabilis</em> IFO 3849</td>
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<td>&gt;250</td>
<td></td>
</tr>
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<td><em>Proteus vulgaris</em> IID OX-19</td>
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<td></td>
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<tr>
<td><em>Salmonella typhimurium</em> IFO 13245</td>
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<td>&gt;250</td>
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</table>