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A hypothesis on peculiar pharmacological behaviour of biologically active natural compounds

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ABSTRACT

A hypothesis is presented to explain the bioactivity of natural extracts, often higher than that of the synthetic bioactive components. This view is different from that based on synergy of the different chemical entities present in the natural substrate. It is based on the occurrence of molecular interactions originating from the high complexity of the natural matrix, following the rules of supramolecular chemistry. The formation of complexes between the active compound and other molecules (or oligoelements) present in the natural environment (leading to conformations more effective for interaction with the receptor) may be justified by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) data. Examples are presented and discussed, suggesting that differences observed between the spectra of the pure compounds and those due to the same molecular species in the natural extracts may depend on some interactions with other molecular species or interactions with oligoelements.

KEYWORDS: bioactivity, molecular interactions, nuclear magnetic resonance, supramolecular chemistry, bimolecular complexes

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1. INTRODUCTION

The early interactions of humans with the natural environment have led to the identification of pharmacologically active substances which, for millennia, were the only medicine available for care, illness treatments and for enhancing either physiological or psychological status. It is not fortuitous that the word “drug”, today used to mention a medicinal substance, derives from the Dutch *droog* (“dried material”) to indicate the *medicamenta simplicia*, *i.e.* natural substances that since the ancient times have been obtained from the natural world and used for therapeutic purposes.

The development of organic chemistry and the investigation on biochemical mechanisms, together with a deeper knowledge of biology and pathophysiology has led, in the last three centuries, to a different approach based on the synthesis of new biologically active molecular species that are not available in the natural environment.

Natural products played and continue to play a highly significant role in drug discovery and development processes [1-5]. When a new biologically active natural product is identified, a general approach is to consider its framework as a starting point to which a series of structural modifications can be carried out. This approach leads to a series of similar compounds whose potential biological activity has to be tested. This is usually performed

by combinatorial chemistry and high-throughput screening methods. The combinatorial libraries so obtained contain much of the structural aspects of the original natural product. This process has been called “Diversity Oriented Syntheses” or “Natural Product Mimics” [6]. By this approach hundreds of new active substances (NASs) also called new chemical entities (NCEs) have been synthesized [4, 5].

Another unexpected method of discovering new potential drugs comes from modern bioinformatics data-mining systems applied to ancient herbal texts [7], which reveal to be a rich pre-existing resource documenting ethnobotanical uses of herbs, containing bioactive compounds possibly to be used as novel pharmacological agents. The multidisciplinary approach to drug discovery, involving the generation of new molecules from natural product sources seems to be an attractive solution to the current productivity crisis facing the scientific community engaged in drug discovery and development.

From the drug–receptor interaction paradigm (as Paul Ehrlich’s aphorism suggested already in the early 20th century: *corpora non agunt nisi fixata*), it follows that the research is addressed to find new components, more active than the natural one, able to promote a more effective interaction with the receptor site. However in phytotherapy it is common to observe that for equal amounts of the active component, the natural extract exhibits a biological activity higher than that of the pure (extracted or synthesized) compound. Williamson [8], in his review ‘Synergy and other interactions in phytomedicines’ underlines that synergistic interactions are the basis to explain the efficacy of low doses of the active constituents present in a herbal product. Wagner [6] reports that the mechanism of action of herbal drugs and their extract preparations, which differ in many respects from that of synthetic drugs or single substances, can be characterized as a polyvalent action and interpreted as additive or, in some cases, potentiating, *i.e.* synergic. This synergy [9] is observed not only in extracts from a single species, but can become more enhanced in mixed-herb formulations [10-12]; these data have been well supported by clinical studies [13-16].

Can the higher activity of natural extracts be explained only in terms of synergistic effects or should some other aspects be considered? It must be emphasized that life is based on molecular interactions. In an interesting recent guest editorial in ‘Accounts of Chemical Research’, [17] the origin of chemical evolution and the essential basis for life development, have been discussed: “Chemical evolution includes the capture, mutation, and propagation of molecular information and can be manifested as coordinated chemical networks that adapt to environmental change. […] A dynamic exchange of network component structures and assemblies, via both covalent and noncovalent associations, is fundamental for the network’s ability to learn, to capture and integrate information about an environment that ensures the network’s future response to similar conditions, as an inherent part of chemical evolution”. Therefore every biological process can be considered as a multitude of proteins, nucleic acids, carbohydrates, hormones, lipids, and cofactors, binding to and modifying each other, forming complex frameworks and assemblies, and catalyzing reactions.

In this view, the idea that the biological activity of a natural extract is due to one (or more) of its component is surely reductive, because it does not take into account all the possible interactions existing among their components. A natural extract is a mixture of hundreds of neutral organic molecules, organic ions, oligoelements and inorganic salts. It must be necessarily assumed that they interact with each other, following the rules of the interactions between molecular units [18].

Just to give an idea of the bond strength present in weak intermolecular interactions, the dissociation energies related to covalent bond, hydrogen bond, dipole-dipole interactions and London (van der Waals) interactions are reported in Table 1 [19]. This comparison must be considered as only approximate, because the actual relative bond strength depends on the molecule involved. A thorough review regarding the molecular interactions existing between partner molecules has appeared in literature [20].

The weak interactions, not leading to the formation of covalent bonds, are relevant in the chemical and biochemical worlds, leading to a new sector of synthetic chemistry, called supramolecular

Table 1. Relative strength of forces (This comparison must be considered only approximate – the actual relative strengths will vary depending on the molecules involved).

Bond type	Dissociation energy (kcal/mol)
Covalent	400
Hydrogen bonds	12-16
Dipole–dipole	0.5-2
London (van der Waals) Forces	< 1

chemistry [21]. It focuses on the chemical systems made up of a discrete number of assembled molecular subunits or components. Traditional chemistry focuses on chemical reactivity of molecular systems, based on cleavages and/or formations of covalent bonds. Supramolecular chemistry examines the formation of weaker non-covalent interactions between molecules. These interactions are based on the above-described forces (*e.g.* hydrogen bonding, metal coordination, π-π interactions) and the studies on supramolecular chemistry have shown the occurrence of folding, molecular self-assembling, host-guest chemistry, molecular recognition and mechanically-interlocked architectures [22]. The emergence of supramolecular chemistry led chemists to effectively prepare structures of different shapes and dimensions.

Also in this case, the supramolecular chemistry approach was inspired primarily by Nature, which displays a wide variety of complex structures based on non-covalent bonding with astonishing precision [23, 24]. The production of these natural structures occurs through molecular self-assembly processes, *i.e.* the molecular system is produced without the guidance of an external source. In other words, due to their chemical nature, (*e.g.* molecular geometry, polarity and presence of specific groups) the original molecules are directed to assemble through non-covalent interactions. Naturally occurring DNA is the best-known self-assembling structure in biological systems [25]. It exists in a double helical form and the two single strands are held together by a number of hydrogen bonds, involving acidic hydrogen atoms (H donors), oxygen and nitrogen atoms (H acceptors) of the purine and pyrimidine bases in order to maintain the double helical structure [25]. Other remarkable examples of naturally occurring nanostructures based on non-covalent interactions are given by the tobacco mosaic virus and the α-helix and β-pleated sheet derived from polypeptide chains depending on chain conformations [25]. Finally it must be emphasized that the tertiary and quaternary protein structures originate through weak intramolecular interactions.

From the above considerations it follows that the enhanced activity of a natural extract, higher than that expressed by the single active component, could be ascribed to the presence of non-covalent complexes of the active component with other species present in the natural substrate.

This behavior may be rationalized by the key-lock interaction between the active component and the biological receptor. It is well known (and this is the basis of the modern pharmaceutical chemistry) that the active component must have a structure (the *pharmacophore* [26]) suitable for the interaction with the target receptor. Molecular modeling studies are usually employed for studying this phenomenon and only the right "key" (*i.e.* the right structure) can interact specifically with the "lock" (receptor). This view is in principle valid, but does not take into account that the structure of the active component is not rigid: at room temperature a molecule can assume different conformations, due to its roto-vibrational excitations. A molecular vibration occurs when the atoms in a molecule are in periodic motion (vibrational frequency). The typical frequencies of molecular vibration are in the range 10^{12}-10^{14} Hz. In this view the "key" cannot be considered fixed, because it can assume different shapes: only when it is in the shape (*i.e.* structural conformation) suitable for the "lock", the drug-receptor interaction can occur.

The drug-receptor interaction must be consequently considered under two different dynamic points of view: on one hand the drug molecule must be

oriented in the right direction *vs* the receptor, on the other its structural conformation (among the many different ones due to its vibrational excitation) must be the right one.

If we consider now the above hypothesis done for bioactive natural products, *i.e.* their interactions with other molecules or oligoelements, it must be considered that this "complexation" will lead to a "freezing" (*i.e.* rigidity) of the molecular structure in a well-defined conformation, with the consequent reduction of the molecule's degrees of freedom. This conformation may behave, as it will be discussed below, as the most appropriate structure for the interaction with the receptor.

Just to give an example of what is considered above, the structures of free EDTA and EDTA-Cu complex are illustrated in Fig. 1. The conformation of free EDTA (in Fig. 1, the structures at the lowest internal energy are given) is completely changed by the presence of a Cu (II) ion: it is evident at first sight that the possible interactions at receptor level of the two structures will be strongly different.

2. MATERIALS AND METHODS

Freeze-dried herbal extracts of artichoke leaves (*Cynara scolymus*), sage leaves (*Salvia officinalis*) and tea leaves (*Chinese Green Tea*) were provided by Aboca SpA (Sansepolcro, Italy) and were produced according to the extraction procedure described elsewhere [30]. The freeze-dried extracts were stored in vacuum at 4 °C until analysis. 2-10 mg of each extract was dissolved in 0.6 mL of H_2O-d_2 (Sigma-Aldrich) and submitted to ^1H and ^{13}C NMR. Spectra were recorded at 27 °C with a Bruker AMX-300 instrument equipped with a multinuclear probe head with z-gradient. TOPSPIN version 3.2 software was used for spectra acquisition and processing.

3. RESULTS AND DISCUSSION

3.1. Some experimental data supporting the hypothesis

The above discussion can be summarized as follows: in a natural extract, due to the presence of hundreds of different molecular species, interactions among them can easily occur, with the formation of molecule-molecule and molecule-oligoelement complexes which must be considered actually responsible for the biological activity exhibited by the extract itself.

Experimental evidences on the molecule-molecule interaction have been described by Tsutsumi *et al.* concerning the caffeine-catechin complex molecule [27]. Both caffeine and (-)-epigallocathechin gallate (EGCg) are recognized as major components of green tea [28]. On cooling down a hot tea infusion, formation of a white-brown precipitate usually occurs (referred to as 'creaming reaction'). Trying to mimic such phenomenon, an aqueous solution of caffeine was poured into an aqueous solution of a selected catechin (*i.e.* EGCg) affording a sticky precipitate, from which a 2:2 complex of EGCg and caffeine was structurally identified by X-ray single crystal structure determination (Fig. 2) [27]. The formation of such supramolecular entities was confirmed to occur also in aqueous solution by ^1H and ^{13}C NMR experiments [29].

In a previous investigation focused on the identification of the metabolomic fingerprint of plant extracts [30] we observed that the ^1H-NMR spectra of *Cynara scolimus* and *Salvia Officinalis* extracts exhibited the characteristic signals of chlorogenic acid and rosmarinic acid, respectively. In the present study we have investigated further these findings and the hypothetical occurrence of molecular interactions in aqueous solutions of three natural extracts of *Chinese Green Tea*, *Cynara Scolimus and Salvia Officinalis* provided by Aboca. Their NMR profiles have been compared with those of pure caffeine, pure chlorogenic acid and pure rosmarinic acid, respectively, recognized as major active components of the three natural extracts. Scrupulous attention was imparted to collect spectra under identical experimental conditions (*e.g.* concentration, temperature, solvent and NMR parameters). The proton NMR spectrum of pure caffeine compared with that of *Chinese Green Tea* extract, both collected in D_2O, is illustrated in Fig. 3. In the upper trace, the methyl groups of pure caffeine (**2**, **5** and **7**) appear as three separate singlets in the 3-4 ppm region, whereas the unique aromatic proton (**1**) gives another singlet at ca. 8 ppm. Bearing in mind that caffeine is a major component in tea extracts, the search of proton signals of caffeine in the *Chinese Green Tea* extract reveals that both

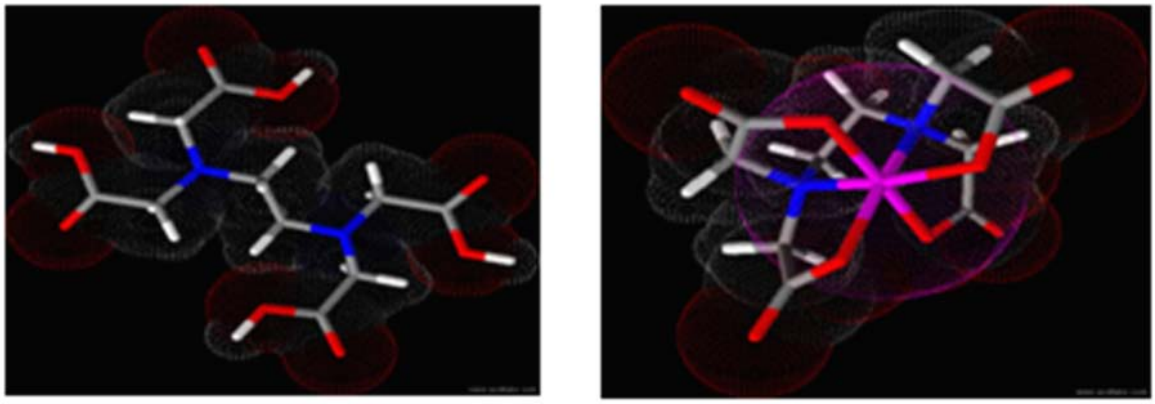

Fig. 1. Structures of EDTA (left) and EDTA-Cu (II) complex (right).

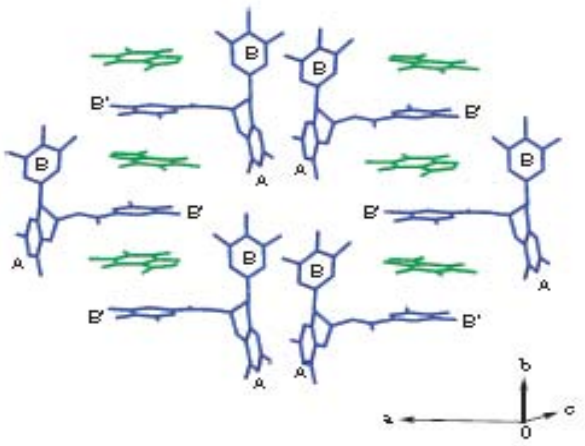

Fig. 2. Molecular structure of the 2:2 complex of EGC:caffeine. EGCg: blue trace, caffeine: green trace. (Reprinted from [27] Tsutsumi, H., Takashi, S. and Takashi, I. 2012, Chem. Lett., 41, 1669 with permission from 'The Chemical Society of Japan').

aromatic and aliphatic signals experience significant shifts.

Analogously, the comparison of the proton spectra of pure rosmarinic acid matched with that of the extract is outlined in Fig. 4. The ^{1}H-NMR spectrum of rosmarinic acid (Extrasynthese) in D_2O shows two aliphatic proton signals (methyne **15** and methylene **16** in the upper trace of Fig. 4) and a more complex set of aromatic protons comprised between vinyl signals **7** and **8**. *Salvia officinalis* extract (Aboca) shows, among others, signals undoubtedly ascribed to rosmarinic acid. These signals (lower trace of Fig. 4) undergo slight to moderate shifts compared to the corresponding signals in the pure synthetic compound. In particular, vinyl protons **7** and **8** (red circle in the molecular sketch of Fig. 4) experience a slight downfield shift, whereas methyne and methylene protons **15** and **16** (blue ellipse in the molecular sketch of Fig. 4) are spread over a larger and not well-resolved ppm region.

These data indicate that the magnetic environment of caffeine and rosmarinic acid, or at least portions of these molecules are affected changing from a batch of chemically synthetized compound to the phytocomplex. The poor solubility of rosmarinic acid in D_2O does not allow the collection of satisfactory carbon spectra to confirm such behavior with a different NMR probe.

The better water solubility of chlorogenic acid (Sigma-Aldrich) provides an additional opportunity to analyze such phenomenon. Again the signal profile of the *Cynara scolimus* batch (Aboca) including chlorogenic acid, displays NMR proton signals slightly different compared to that shown by a pure synthetic batch of chlorogenic acid (data not shown). Moreover, the low field 110-190 ppm region of the ^{13}C{^{1}H} spectrum of pure chlorogenic acid (upper trace of Fig. 5) shows characteristic signals of the carboxylic carbon **1′** and of the vinyl carbons **7** and **8**. In the extract (lower trace of Fig. 5), the carboxylic signal disappears, likely shifted into the carboxylic/carboxylate-rich region in the extract beyond 180 ppm, whereas vinyl carbons experience a marked variation of the signal intensity.

Overall, data arising from both rosmarinic acid/*Salvia officinalis* and chlorogenic acid/*Cynara scolimus*

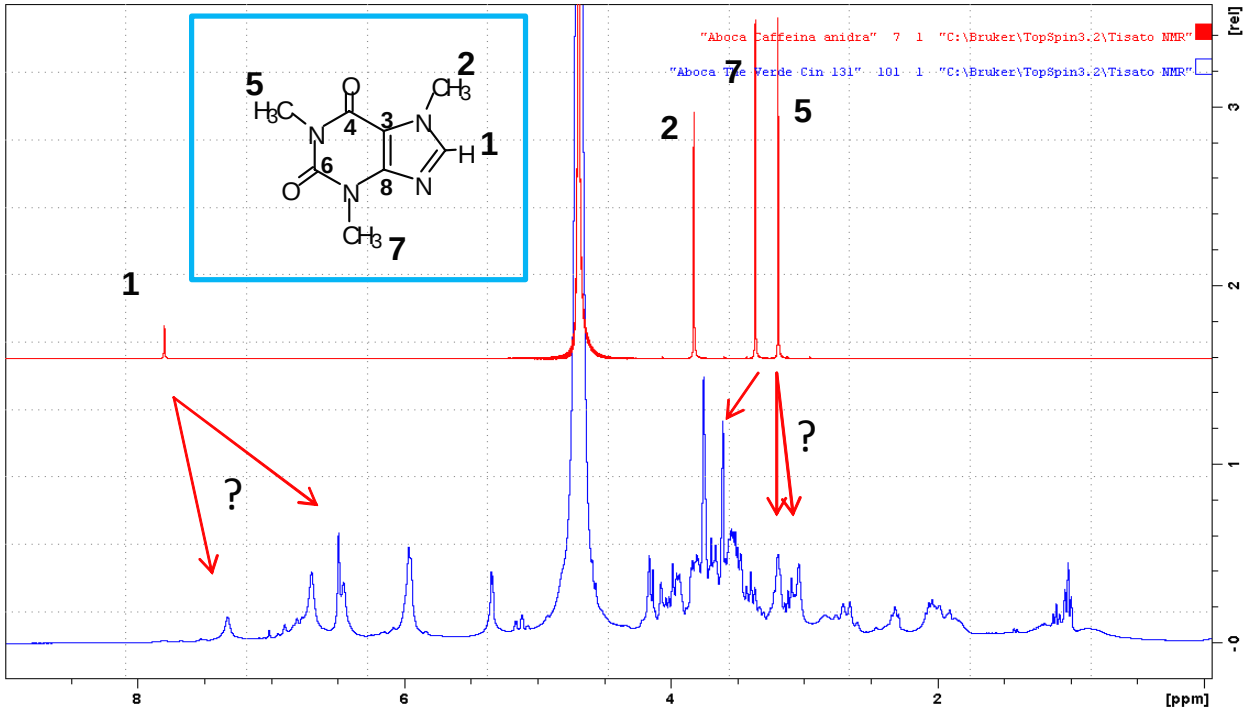

Fig. 3. ^{1}H NMR spectrum of pure caffeine (Chicaffè; upper trace) compared with that of *Chinese Green Tea* (Aboca; lower trace).

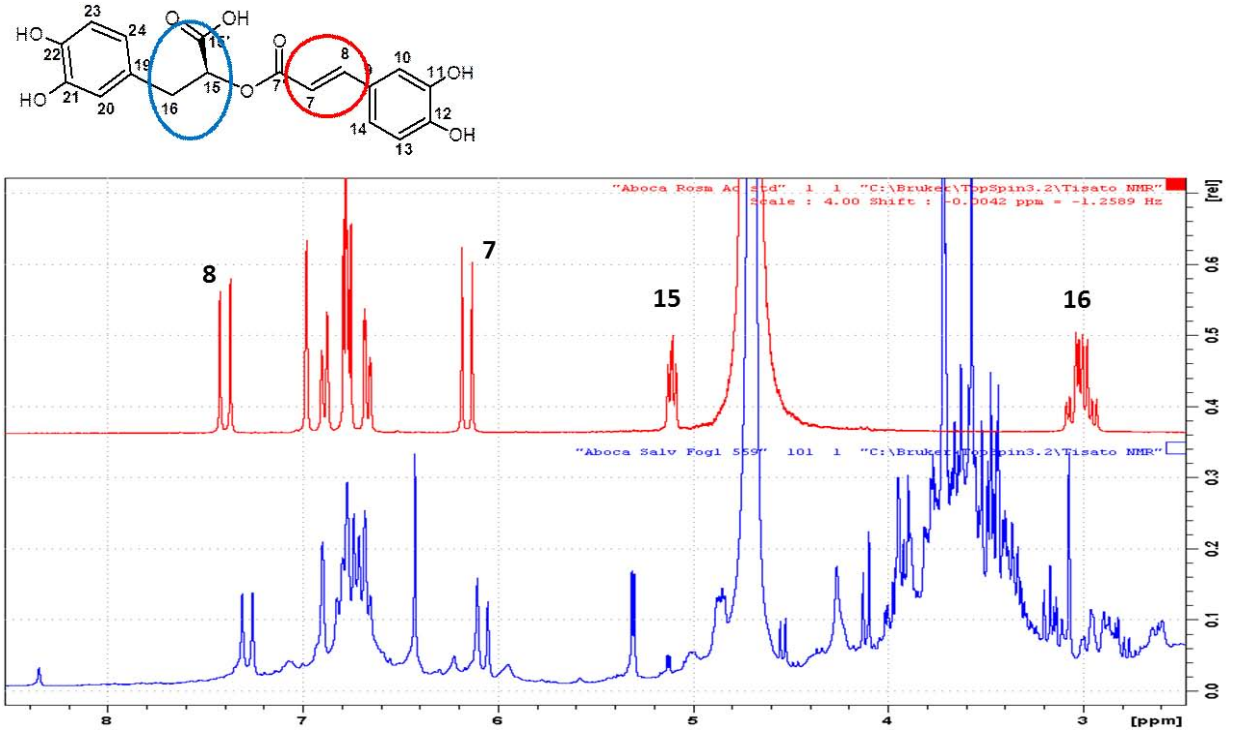

Fig. 4. ^{1}H NMR spectrum of pure rosmarinic acid (Extrasynthese; upper trace) compared with that of *Salvia officinalis* (Aboca; lower trace).

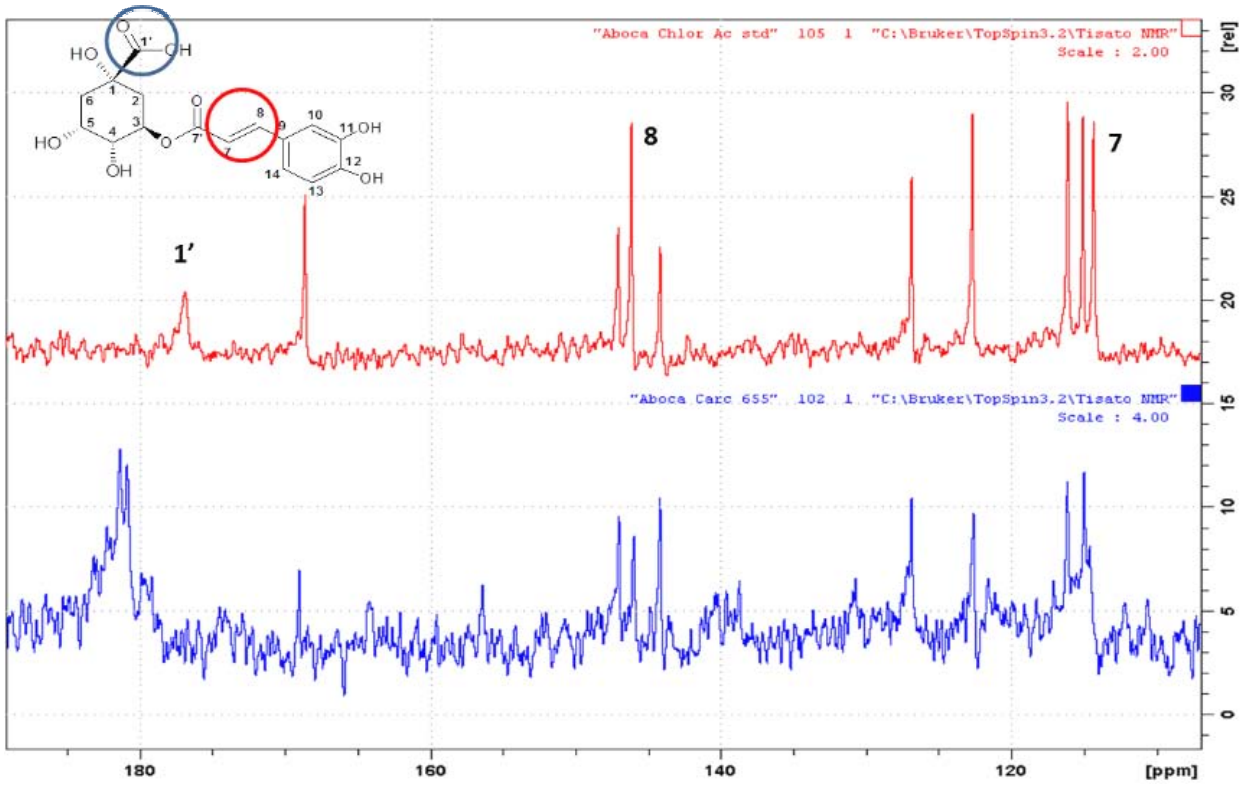

Fig. 5. $^{13}C\{^{1}H\}$ NMR spectrum in the low field 110-190 ppm region of pure chlorogenic acid (Sigma-Aldrich; upper trace) compared with that of *Cynara scolimus* extract (Aboca; lower trace).

comparison support the hypothesis that polyphenolic species (rosmarinic and chlorogenic acids) comprised in the phytocomplex are subjected to molecular interactions that are not present in the synthetic, pure batch and are therefore peculiar of the extracts. In both cases, these interactions modify appreciably the magnetic environments near the vinyl and the carboxylic groups (red and blue circles, respectively, in the molecular sketches of Figs. 4 and 5).

3.2. Evolutionary pharmacology?

The experimental data reported and discussed above lend support to the presence of molecular interactions inside a natural extract, interactions which lead to molecular structural conformations different to those present in synthetic products.

This reflects on more effective interactions between active molecule and receptor due to the reduction of one of the two dynamic processes influencing this interaction; in fact while the orientation of the molecular species *vs* the receptor still remains a relevant point, the rigidity of the active molecule due to its complexation with other molecules or oligoelements strongly reduces (or, in the best conditions, excludes) all the dynamic problems related to molecular vibrational excitation. At this point a question arises: when and how was the receptor site been developed? In our opinion it is reasonable to assume that it has been structured over tenths of millennia as a function of the structural conformation of the molecular species experienced by the living organisms in the natural environment, leading to the development of specific sites at protein level, responsible for their transport and metabolism.

According to Darwinian theory, a combination of mutation, selection and drift has led to the origin of phenotypes with peculiar characteristics [31]. It has been demonstrated theoretically, with the aid of computer simulation but also following laboratory experiments, that the genesis of a functionally adapted protein conformation can be the result of selective pressures. These produced the fixation of

the characters from an ancestral population, as the result of a few mutations of large effect, or, alternatively, by sequential accumulation of many mutations of small effect [32]. Whatever were the evolutionary patterns that led to the origin of a given receptor structure, the present-day conformation of the receptor is consequence of multiform interactions with endogenous and exogenous ligands. The presence of a receptor in an organism is the latest result of several orders of natural selection interactions, and just under the probabilistic point of view, its responsiveness is physiologically more feasible for a natural occurring structure.

3.3. Bioavailability

A pivotal pharmacokinetic property of a drug is the bioavailability, defined as the fraction of an administered dose (in form of unchanged substance) that arrives into the systemic circulation, thereafter available for its direct use by the organism or for storage into accumulation sites [33]. The bioavailability can be affected by several factors, some linked to the physico-chemical properties of the substance, others strictly dependent on the formulation, and many others due to individual phenotypic or genetic aspects. Bioavailability greatly influences the onset of the therapeutic effect and in many cases it can also lead to adverse effects (due to hyper/hypo-dosage).

It must be remembered that the transmembrane transport mechanisms of a drug (natural or synthetic) is mediated either by passive transport or by active mechanisms, including diffusion through the lipid bilayer, hydrophilic pore diffusion, facilitated carrier systems, and true receptor systems. In all these situations, the already discussed factors linked to supramolecular chemistry and determining the activity of a molecule can also influence its bioavailability. Chirality in membrane lipids has already been reported as a possible determinant in modulating drug interaction and hence drug effect [34].

Within the formulation aspect, the role of excipients (also seen under the dualism, natural/synthetic) in influencing bioavailability is of enormous relevance, as highlighted by the still widely debated topic of relative bioavailability or bioequivalence, which refers to the comparative evaluation of bioavailability from two different formulations or products that contain the same compound. From this point of view, botanical extracts, due to their high complexity and occurrence of the above-described phenomena, can be considered highly effective in influencing the mechanisms of transport across the biological membranes (gut, skin, mucosal membranes).

4. CONCLUSIONS

The hypothesis proposed to justify the higher bioactivity of natural extracts, based on the presence of complexes between the active compound and other molecules (or oligoelements) present in the natural environment (exhibiting conformations more effective for the interaction with the receptor) appears to be justified by ^{1}H and ^{13}C nuclear magnetic resonance data. The differences observed between the spectra of the synthetic pure compounds and of those present in the natural extracts indicate that in the latter case some interactions with other molecular species or with oligoelements are present. These results are also in agreement with the systemic view of a natural substrate that must be considered not only a mixture of different components, each of which exhibiting peculiar properties, but a system in which numerous and continuous intermolecular interactions occur. These interactions can also lead to the formation of molecular entities that are stable from the thermodynamic point of view. These findings will be investigated in the near future to obtain further experimental evidences supporting this behavior, which necessarily reflect the real pharmacological properties of natural extracts.

CONFLICT OF INTEREST STATEMENT

Authors declare no conflict of interest.

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