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Assessing the Role of African Indigenous Vegetables to Improve Agriculture Sustainability and Diet Quality

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Index

1. Towards a healthier and more sustainable food consumption

The modern global food system is affected by a series of flaws dealing both with an unsustainable food production and with the healthiness of consumed food. This last issue is pushed beyond safe boundaries in terms of balance between nutrients and with unavoidable consequences for human health (Willett et al., 2019). Such situation is destined to become even more worrying if we consider that the global population is going to rise up to more than 10 billion people by 2050 and that this increase will even more pressure the environment and the global exploitation of natural resources (Mittal & Gupta, 2015). Since the beginning of the 2000s the so called "Earth Overshoot Day", that is the moment in the year starting from which humanity has wasted the amount of resources made available by Earth for the ongoing year, is estimated to occur in August instead of on the 31st December: this means that each year we humans waste on average 1/3 more resources than those we could exploit (Fig. 1) Also in 2020, when the outbreak of COVID-19 pandemic should have lowered human footprint on Earth, the date occurred on the 22nd August (Fig. 1), denouncing a general over-exploitation of resources that is a problem of primary concern to guarantee a safe and balanced living for human mankind on the planet .

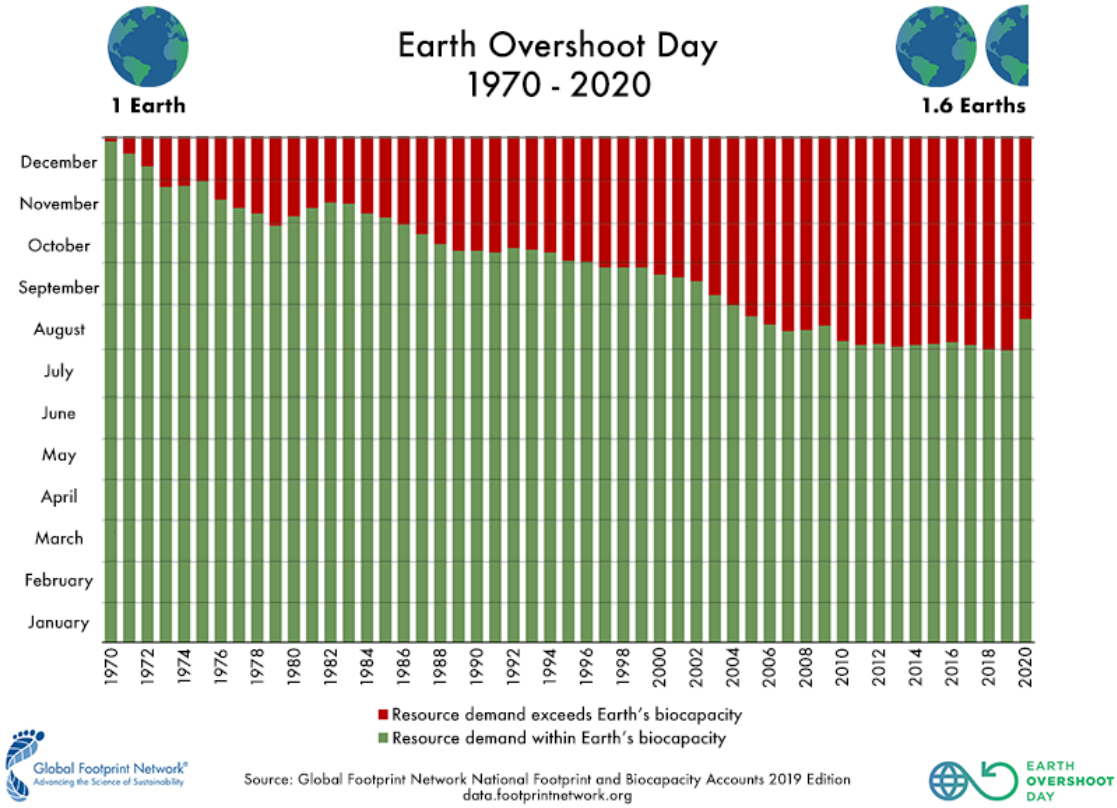

Fig. 1: From 1970 to 2020 the advent of the Earth Overshoot day is even more anticipating. In 2020, the date was 22nd August. To be able to afford a level of resource exploitation such as that of 2020, humanity would need to be provided of 1.6 planet instead of 1. Image source: Overshootday.org

Food is one of the most important aspects dealing with resource exploitation and waste, environment, and human well-being, since food requirement is the major driver of land exploitation (Kanianska, 2016). Moreover, the adoption of water wasting and pollutant food supply chains (such as the cultivation of many common cash crops as well as many kinds of livestock) are widely responsible both for the

lack of ecological sustainability and for dietary-related issues all over the world (Willett et al., 2019). Generally, the modern food system is characterized by the prevalence of dietary patterns including red meat, starchy vegetables and sugar (Fig. 2) associated with a low intake of micronutrients, such as calcium, iron, zinc, magnesium and vitamins. Therefore, the consumption of a high rate of animal-derived food sources is needed to be balanced with the provision of further dietary patterns, particularly those of plant origin, in order to prevent micronutrient deficiency derived pathologies such as stunting, famines and a wide set of non-communicable diseases (NCDs) such as diabetes, hypertension, neurodegenerative disorders, cancer and chronical respiratory diseases (Willett et al., 2019).

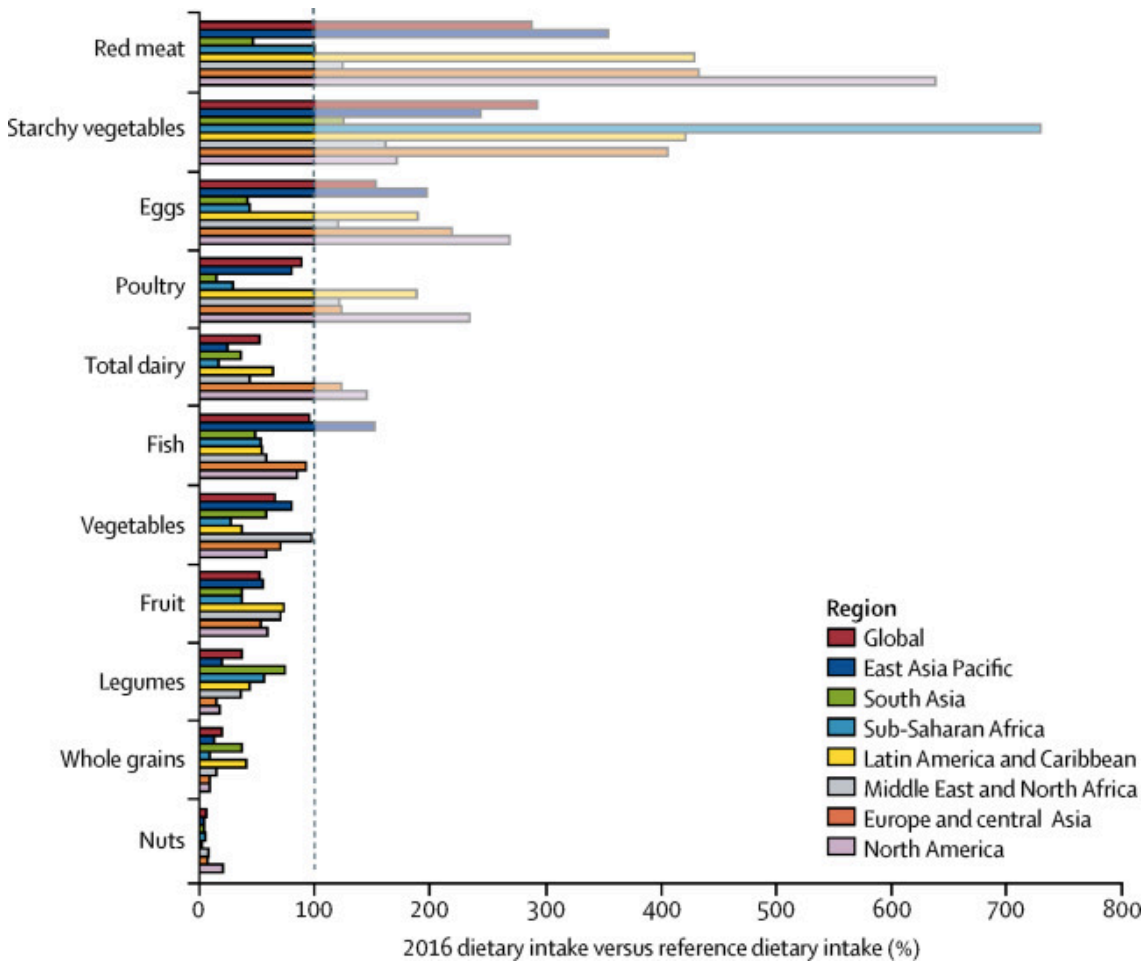

Fig. 2: The *EU*-Lancet commission found that in 2016 the dietary patterns mainly consumed worldwide were red meat, starchy vegetables and, to a less extent, eggs and poultry. The West hold the primacy in the consumption of these animal-derived products. Conversely, plant-derived dietary patterns were found significantly below the minimum recommended level to support human diet requirements. Image source: Willett et al., 2019.

Plants are an extraordinary source of microelements and bioactive compounds whose provision in the diet is known to significantly lower the predisposition to many NCDs and to attenuate stunting risk (Sivakumar et al., 2018). Plants derived foods represent different dietary patterns with peculiar nutritional and nutraceutical features. Many edible plants are great sources of micronutrients (e.g., zinc, iron, manganese, magnesium, calcium), vitamins (retinal, niacin, folic acid, ascorbate and tocopherol), fibers with prebiotic properties and secondary compounds (i.e., terpenes, phenols, betalains, alkaloids) responsible for several nutraceutical properties (e.g., anti-diabetic, anti-hypertensive, antioxidant and anti-inflammatory, anti-cancer and antiaging) (Natesh et al., 2017). Among angiosperms, there are families and genera synthetizing peculiar metabolites. For example, legumes are source of folates, fibers, phenols, calcium, iron, amino-acids and proteins (Sparvoli et al., 2015). There are also plant organs rich in specific classes of nutrients. For instance, nuts are associated to a great uptake of unsaturated fatty acids such as the omega-3 and omega-6 as well as tocopherols and minerals (Ros, 2010), just to cite the most important plant dietary patterns.

Evidences show that the provision of an increased amount of plant derived foods would be a good candidate to address the challenge of a more sustainable food production in the Anthropocene era, not only for the above mentioned plant properties but also because their cultivation may be much more sustainable if thought to attenuate the overspread of livestock which requires a significant amount of natural resources and is responsible for about 14.5% of total anthropogenic greenhouse gases emissions (Grossi et al., 2019). However, the modern agriculture is practiced in a non-sustainable manner; therefore, it is

necessary to focus both on plant biodiversity and on cultivation strategies to identify more sustainable agri-food production systems.

After the Green Revolution of the 1970s, the entire agronomic food system has focused on small groups of crop species belonging to a few plant families (e.g., Poaceae) which provides for more than 60% of the total cultivated species in the world (Cheng et al., 2017). This occurred because the strategy adopted to maximize yields was directed to a minimum range of species to obtain the best results in terms of food provision as a consequence of the increasing demand. While providing for these improvements, the concept of biodiversity as an ally of a healthy nutrition was progressively lost and, with it, also the sustainability of the food production started to be neglected (Pingali, 2012). Unfortunately, the domesticated crops, improved during the Green Revolution, are unsustainable nowadays. Most modern crops are highly water demanding, scarcely able to defend against pests and pathogens and many of them require soil treatments such as fertilization and tillage practices. These requirements have a high environmental cost in term of resource wasting (water, energy) and carbon footprint (Mateo-Sagasta et al., 2017). Conversely, many local and neglected plant species, well adapted to harsh environments, are less requiring in terms of cultivation and may provide a valid alternative to reinvent agriculture in a way that is compliant with both the current climate situation and the nutritional demand of the modern citizen (e.g., less sugars and fats). This condition could also contribute to mitigate the lack of micronutrients (vitamins, Ca, Fe, Mg, Zn), that afflicts people diet in many regions of the world from Sub-Saharan Africa to Western countries (Maseko et al., 2018).

The rediscovery of traditional and spontaneous biodiversity as a source of global food production may be a promoter of a more sustainable food system. As claimed

by recent studies, the identification of proto-domestic plant species (i.e., rustic and competitive plants, resistant to biotic and abiotic stresses) with high nutritional proprieties may be a driver for the introduction of new promising species at the global agronomic system, shadowing the traditional but less sustainable ongoing panel of exploited crop species (Cheng et al., 2017).

Another challenge of the modern food production systems is the fact that the current soil exploitation is becoming year by year even more pressing (e.g., erosion and leaching), with important consequences in terms of habitat and biodiversity loss (IPCC, 2019). Soil exploitation is also related to many factors, such as cities expansion. However, the low productivity of some crops combined with the growing demand for agricultural products enhances the amount of cultivated land that each year steals soil to natural habitats (IPCC, 2019). A glaring example of this trend is shown by deforestation. Amazon forest loses every year hundreds of thousands ha (MAAP Synthesis, 2020) and similar situations are occurring in many other regions of the world, such as South Africa and South East Asia (Estoque et al., 2019; McNicol et al., 2018). Soils deprived of the forests are converted to cultivated lands or exploited for livestock, with a consequent worrying increase of the carbon footprint (Hoefle, 2012). There is therefore an urgent need to reconsider the modern food systems also to address the topic of land consumption in a more global view that impacts all the biogeochemical cycles, such as those provided by SDG 15 ("life on land") of the UN Agenda 2030. At the same time, the topic of SDG 2 – "zero hunger"- represents a priority especially for developing countries. Therefore, a comprehensive effort to reconsider the modern food system requires many elements (e.g. soil, biodiversity and water) and actors. In this context, the role played by science is of primary concern in order to integrate many issues, starting

from agroecology principles to the nutritional properties of food products to prevent the outbreak of NCDs.

2. The value of plant biodiversity and the role of indigenous vegetables

A first mitigation strategy to deal with the modern general unsustainability of the agricultural systems is to look after those environments characterized by a high level of spontaneous and proto-domestic plant species which were only partly interested by the effects of the Green Revolution. The areas of the planet that have been less subjected to anthropogenic pressure following the Green Revolution, such as Sub-Saharan countries, represent an interesting place for finding new species for the modern society (Jama & Pizarro, 2008). These contexts are a great source of plant biodiversity that may be of interest for multiple purposes: i) at the agronomic and environmental level, the rediscovery of marginal and traditional plant species may become a valuable alternative to the main few intensively cultivated crops of high carbon footprint; ii) at the health level, the adoption of a more diversified plant diet is an important feature dealing with food safety and nutritional prevention (Maseko et al., 2018). In most cases, these local species show a very limited cultivation area and sometimes are consumed only by local populations (Meseko et al., 2018). These species have been also known in literature as "Neglected and Underutilized Species" or more appropriately "African indigenous vegetables" (hereafter AIVs) since the majority of them is of African origins and was domesticated in the context of Sub-Saharan African environments (Grubben et al., 2014). Nowadays the term is also used to refer to species that are considered marginal in the local agricultural food systems, supplanted by the advent of "Western" staple crops, such as maize, wheat and rice (Maseko et al., 2020). Therefore, AIVs survive in the context of small-holding farms and local districts where they best show their suitability to non-intensive agronomical

contexts (i.e., no tillage, low fertilization, low water demand). Because of their progressive decline due to the spread of Western staple crops, their cultivation needs to be enforced in several African regions. In these contexts, some societies and organizations are acting to raise awareness of the role of vegetables for improved health and global poverty alleviation (Ebert, 2014). An example is the World Vegetable Center (AVRDC, https://avrdc.org/) in Arusha (Tanzania) which sells to local farmers seeds of these species to encourage their adoption in the local agricultural systems (Fig. 3).

Fig. 3: At the World Vegetable Center (AVRDC) of Arusha (Tanzania) African Indigenous Vegetables are cultivated and breeded to gain the most resistant and yielding varieties to be sold to local farmers in order to encourage their adoption into the local agricultural systems.

However, these plants are not only a resource for local populations, but they represent a value for the entire global agriculture. The panel of species belonging to AIVs is wide and composed of a heterogenous class of species that show good resistance to environmental stressors (e.g., drought, heat) and interesting

nutritional profiles (e.g., minerals, vitamins and phytochemicals in leafy crops and proteins, aminoacids and unsaturated fatty acids in seed and grains). Among the most promising species of AIVs there are many species belonging to the family Fabaceae (Duodu et al., 2017). For example, members of *Vigna* genus (e.g., *V. radiata* (L.) R. Wilczek, *V. unguiculata* (L.) Walp., *V. subterranea* L.) are well known as a source of proteins (20-40% of the total seed weight) and aminoacids, as well as micronutrients (calcium, zinc, manganese, iron) and vitamins (tocopherols, niacin, folates, ascorbate). Moreover, these species are also rich in phytochemicals such as polyphenols, alkaloids and saponins (Harouna et al., 2018). Other relevant legume species are *Cajanus cajan* L. and *Lablab purpureus* (L.) Sweet, of interest for the great aminoacidic composition of their seeds with a high ratio between essential and total aminoacids (Hossain et al., 2016; Oshodi et al., 2009). It should also be acknowledged that several AIVs are leafy vegetables rich in essential microelements such as vitamins and minerals. Among these, *Cleome gynandra* L., *Corchorus olitorius* L., *Solanum nigrum* L., *Amaranthus* spp. are considered very promising alternatives to prevent malnutrition in many countries of the world. These are of interest both for their grains and leaves. Grains contain high amount of unsaturated fatty acids, tocopherols, minerals, while leaves are rich in valuable phytochemicals and vitamins of the B group (niacin, riboflavin, folic acid) (Sivakumar et al., 2018). An accurate balance in the consumption of these marginal but nutritionally valuable species may produce beneficial effects for human nutrition, limiting the spread of micronutrient deficiencies which may be at the basis of the outbreak of many common NCDs also in urbanized countries (Grubben et al., 2014). Moreover, all the edible portions of these species belong to dietary patterns that are consistently below the minimum recommended intake as shown

in Willett et al. (2019). These species also resist better to environmental stressors posed by climate change (i.e., low water availability and high temperatures) and require low level of phytochemical and agronomical treatment in terms of field management (Maseko et al., 2018). Of course, it may be useful to promote adequate breeding programs to ameliorate the agronomic performances of these species (Muhammad et al., 2020) starting from their local diversity. For these reasons, the adoption of African proto-domestic plant biodiversity in the local agricultural systems, also including the Mediterranean area, may be an interesting alternative to counteract the modern intensive agronomic system which is accelerating the impact of climate change and risks to expose the global population to the outbreak of unbalanced nutrients related pathologies.

3. Conservation agriculture: a model for agriculture sustainability

A further mitigation strategy to look after for the reduction of the human footprint in the agronomic context is to identify productive but sustainable ways of cultivating (Adegbeye et al., 2020). The modern agronomic system is concentrated to provide for high yields in order to satisfy the increasing food demand of the growing population (Graham Centre, 2015). In this way, resources such as water and soil are even more subjected to increasing pressure which risks to pose, in the very next future, great concerns in terms of environmental sustainability and food security (Adegbeye et al., 2020). The Green Revolution of the 1970s focused on a low number of crops which have been genetically modified to increase their yields, but the costs required to maintain these species is very high, especially if this limited panel of crops is required to provide food for the entire world population. Moreover, the growth and the yield performance of such crops is linked to the provision of high amounts of fertilizers and agrochemicals and requires an huge amount of water (Rull, 2010). In the last decades, different strategies to deal with the intensive agriculture system were evaluated. Organic farming has become a trend in the modern agriculture and the principles which it is based on deal with the absence of whatever kind of treatment of soil in order to promote the spontaneous growth of crops with no chemical inputs. Moreover, this strategy is often associated to the attempt of restoring the naturality of the environmental context where crops are grown (Reganold & Wachter, 2016). Despite many different beneficial peculiarities, especially in terms of environmental sustainability, there are still some concerns about the adoption of organic farming strategies in the agronomic systems due to the fact that yields are usually below

the expectancies (with negative consequences in terms of market prices) and that the environmental advantages are not always addressed (Tuomitso et al., 2012). More recently, the agronomic systems started to consider the adoption of another strategy alternative to the conventional agriculture: the conservation agriculture approach.

Conservation agriculture is based on three main principles (Fig. 4): (i) direct planting of crops with minimum soil disturbance, (ii) permanent soil cover by crop residues and cover crops, (iii) crop rotation (Kassam et al., 2009). The first strategy deals with the adoption of minimum tillage regime in order to preserve soil structure, a pivotal aspect to maintain an adequate level of ecosystem naturality in the microbial and soil faunistic communities that are involved in different biological properties, such as carbon and nitrogen mass sequestration and processing (Pal & Basak, 2019). The second strategy deals with the necessity to promote rhizosphere maintenance also during the non-growing season of the field (Pittelkow et al., 2015). This leads to a better water permeation within the soil that may guarantee a water saving along the agronomic season. Last, crop rotation which is a principle known since the Middle Ages and that is very important to promote soil fertility (in terms - for instance - of nitrogen and carbon stocks) and to avoid soil depletion over time (USDA, 2015).

Fig. 4: minimum soil disturbance (through the adoption of no tillage practices), permanent soil cover (through the exploitation of suitable cover crop species) and diversified crop rotation over time are the three main pillars of conservation agriculture.

An important aspect dealing with the conservation agriculture approach is that inputs on the field may be provided to ameliorate yield performance and to reduce the impact of pests and parasites and this is one of the main aspects differentiating organic farming from the conservation agriculture approach (Vanlawe et al., 2014). Despite that, many different beneficial aspects may be addressed through the adoption of this approach: first of all, the massive reduction in the usage of mechanization to till soils represent an important attempt to go towards the reduction of greenhouse gases emissions from the agronomic sector. Furthermore, it has been deeply shown that no tilled soils are able to sequestrate a greater amount of carbon (also in the form of carbon dioxide and methane) than till ones and this is due to the better integrity of the microbial communities below ground (Montanaro et al., 2017). Moreover, the maintenance of cover crops along the whole year leads to a better preservation of water. Therefore, water requirements to grow crops is reduced if compared to the demand posed by tilled fields (Boselli et al., 2020). Carbon and nitrogen content in the topmost level of soils are found to be higher under conservative management, even though the effects at the

deepest levels appear controversial (Fiorini et al., 2020). However, agronomists and researchers are still divided about the benefits and drawbacks of conservation agriculture as a sustainable alternative to contrast the negative impact of conventional agronomic management (Pittelkow et al., 2015). In this context, the response in terms of yield, considering not only the most common staple crops but also those marginal but resistant species (such as the African indigenous vegetables), is important to identify the most sustainable strategies and opportunities for the modern food system. Furthermore, there are increasing evidences highlighting that plant metabolism is affected by the environment. In particular, secondary metabolites and peptides are responsible for adaptation responses both to biotic and abiotic stresses, such as pest attacks, interaction with parasites and mutualists as well as drought, thermal and salt tolerance and show consistent variations in their concentration and/or expression when plants are subjected to (Mosolov & Valueva, 2011; Sharma et al., 2019; Ramawat & Goyal, 2020). Since some of the conditions posed by the conservation agriculture approach (such as no tillage or cover crops maintenance) may represent stressful conditions for plants (e.g., cover crops may compete with the principal crop for resource exploitation, no tillage may permit the growth of spontaneous species in the field that again represent resource competitors) it is necessary to identify the crops able to deal with such kind of concerns and to evaluate if the physiological responses triggered by these stressful environmental conditions may elicit some metabolic pathways (such as those involved in the biosynthesis of secondary compounds or aminoacids as osmoregulators) that may improve the nutritional value of crops especially in terms of proteins and micronutrient provision.

4. Legumes to promote sustainable food and agricultural systems

The identification of suitable approaches to increase the sustainability in agricultural food systems requires to identify high-yielding plants in conservation agriculture conditions, compared to conventional management strategies (Zhang et al., 2018). At the same time, they should be able to provide for an adequate amount of nutrients and bioactive compounds to support human health (Bamji et al., 2020). For the satisfaction of these requirements, legumes stand out due to the high level of adaptability to harsh environmental conditions and for the nutritional supply they provide to the diet. This situation contrasts with the fact that in the modern society, the consumption of legumes (and their cultivation) is below the minimum recommended level (Fig. 1). There is therefore the need to reverse this trend by acting both on a social level through the promotion of legume-based products and on a scientific level, by identifying cultivars showing good performances in terms of quality and quantity of nutrients. At the social level, there is the need to turn to the agronomic productive system considering the role of legumes in soil preservation and biodiversity safeguard. Legumes are able to fix atmospheric nitrogen (N_2) at the radical level converting it into the ammonium ion (NH_4^+). In this way, the cultivation of legumes does not require chemical fertilization since the ammonium ion is made organicable into biomolecules such as aminoacids and proteins. In addition, legumes are important crops to be considered and implemented within crops rotation programs and support the requirements of the third conservation agriculture pillar (Stagnaro et al., 2017).

Basing on EU Green Deal programme, it is important to promote strategies of diversification in the agricultural environments. In these contexts, it is known that the best functionality for the ecosystem maintenance is to promote an extensive agriculture approach with crop diversification to be supported also during the same growth season (Grass et al., 2019). So, the usage and the promotion of legume cultivation is not to be intended in the way it is performed (sometimes also illegally) in some regions of the world, such as in the Amazon where the cultivation of soybean (*Glycine max* L.) is responsible for an increasing trend of deforestation and land exploitation and the massive adoption of treatments typical of the conventional agriculture (GMOs, agrochemicals, intensive water exploitation) is a routine (Jia et al., 2020). To support the adoption of legumes into the modern agriculture systems it is needed to take highly into account their adaptability to many different types of climate and their adoption needs to be intended as a way to find crops that naturally may counteract the worrying effects of a changing climate (Stagnaro et al., 2017).

There is another important reason to promote legume production and consumption. Today, the main source of proteins in Western diet is red meat (Willett et al., 2019). The consumption of meat at the global level is consistently higher than other protein sources such as legumes, fish, total dairy, poultry and eggs. North America stands at the top of this ranking, followed by Europe and central Asia (Willett et al., 2019). This has negative effects not only on human health but also on the environment, because livestock practices are among the main sources of greenhouse gases emissions (Grossi et al., 2019). Transition to healthy dietary patterns by 2050 will require substantial dietary shifts, including more than 50% reduction in global consumption of processed food, animal source foods (ASF)

and more than 100% increase in consumption of plant-based foods, including nuts, fruit and vegetables. Although legumes represent an alternative source of proteins and several other micronutrients, in many industrialized countries their consumption does not reach the minimum recommended intake (Willett et al., 2019). Science and technology, together with politics, must therefore propose credible solutions to make legumes an adequate alternative to animal protein resources.

From 1970 to 2013, legumes consumption in Europe has been subjected to a constant decrease due to the tendency to adopt cereal crops characterized by a high market demand, but the paradox is that European countries are forced to import from other continents (such as South America) legumes, especially to satisfy livestock nutritional demand but usually also for food purposes (Zander et al., 2016). In this way the negative impact of land sparing in the wildest regions of the world is constantly supported and this is witnessed by the impressive level of deforestation of the last years in those regions of the world where there is a continuous over-exploitation of soils, devasted by fires and reconverted for agriculture and livestock purposes (Pereira et al., 2020).

However, a transition to a more sustainable diet rich in legumes and a consequent re-adoption of legume crops into the agricultural systems of the West requires a substantial change of the typical “Western” dietary habits and food choices (also supported by industrial stakeholders) in combination with suitable strategies to enhance legumes cultivation, distribution and consumption (Wolk, 2017). This underlines how the concept of sustainability in the food system of our days is inseparable from that of healthiness. The investigation of biodiversity at the genetic, metabolic and protein level may let to discover a wide panel of compounds

of high interest for health and economic purposes: this approach is called bioprospecting and may be useful to be taken it into account while evaluating the dietary patterns that best fulfil nutritional prevention issues (Oyemitan, 2017). Precisely, with regard to healthy properties, legumes are a dietary pattern of high interest for the nutritional supply to the human diet (Fig. 5).

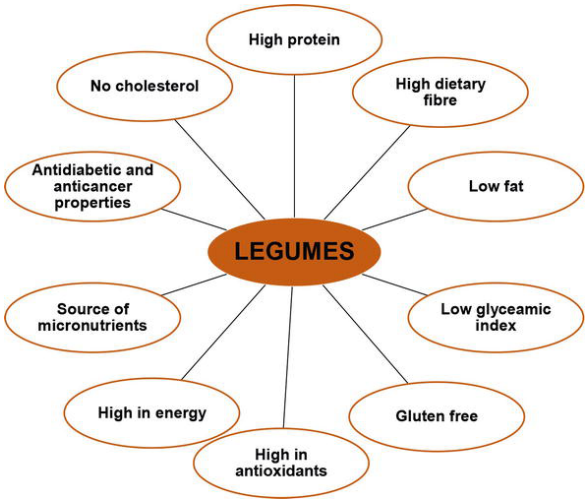

Fig. 5: list of the main nutritional and nutraceutical properties related to legumes consumption. Image source: Maphosa & Jideani, 2016.

Besides their well-known value for the protein and aminoacid content, which makes them a great alternative to the consumption of animal derived items, a recent focus concerns their capability to prevent the outbreak of a wide panel of non-

communicable diseases (Sparvoli, 2015). This makes them suitable to be considered as functional foods. Among the most interesting bioactive compounds we found phenolic compounds (flavonoids, anthocyanins and phenolic acids) which localize mainly in the seed tegument (Giusti et al., 2019). These metabolites exert a wide variety of preventive properties (anti-oxidant, anti-inflammatory, anti-microbial, cancer preventive, neuroprotective, anti-diabetic and blood pressure regulative) (Guaadaoui, et al., 2020) even if sometimes are also considered as anti-nutrients such in the case of tannins which are known to bound proteins and to reduce their digestibility and bioavailability (Gilani et al., 2012). Sometimes the phenolic composition of seeds increases with germination processes and this may also be associated to an increase in the bioactive properties that arise from these metabolic variations. Therefore, it is considered of interest also the consumption of legume sprouts for the nutraceutical properties that they may exert (Teixeira-Guedes et al., 2019). Minor secondary metabolites such as genistein, formonetin, biochanin A isolated in chickpea (*Cicer arietinum* L.) were associated to type 2 diabetes prevention (Lin et al., 2020), resistant starch and dietary fibers from common bean (*Phaseolus vulgaris* L.), group B soyasaponins from lentil (*Lens culinaris* L.) were found to display prebiotic and hypocholesterolemic properties (Kilua et al., 2020; Micioni Di Bonaventura, 2017). Another important class of molecules associated to healthy properties are bioactive peptides. Many of them are proteins such as globulins and albumins that are converted into smaller peptides during the digestion processes. When these peptides are processed into the gastro-intestinal trait, they gain a wide set of bioactive properties (such as anti-inflammatory, anti-oxidant, hypoglycemic, anti-hypertensive, cancer preventive) that they did not have at the entire protein level. So, the processing mediated by digestion is an important

step to increase the functionality of these macromolecules (De Fatima Garcia et al., 2020; Gonzalez-Montoya et al., 2018). Also, protease inhibitors, such as amylase and chymotrypsin inhibitors, have been deeply investigated for their nutraceutical properties in pulses. These peptides are synthetized by plants as anti-nutrients to avoid pest and insects attack, so their biological role is to act as defence mechanisms against biotic stressors. These inhibitors are able to bound target enzymes in insect tissues involved in digestion processes and therefore they enable insects to feed (Stankovich et al., 2020). Many of them have been found to exert bioactive properties especially in terms of cancer prevention (Sparvoli et al., 2015). Moreover, due to their resistance to harsh environmental conditions, such as the low pH of the stomach or the high temperatures which legumes are subjected to during boiling processes, they are very likely to join target tissues. These features increase their bioavailability and make some of these peptides able to exert effectively their cancer preventive properties (Srikanth & Chen, 2016). These evidences are further enforced by the fact that people leaving in the regions of the world where legumes are more consumed (Africa and south Asia) show lower occurrence of some of these malignancies (such as colon cancer) compared to the Western countries where this dietary pattern is widely below the minimum recommended intake (Aranda-Olmedo & Rubio, 2020).

Given all these features, both at the environmental, nutritional and nutraceutical level, the revaluation of legume among the modern agricultural systems seems to be a pivotal aspect to address the aims posed by SDG n.2 and the identification of resistant crops within this clade may be an important trend to address food security in a global perspective. In this context the *Vigna* genus shows some peculiar traits both at the genomic and physiological level that make it suitable to the new

environmental conditions posed by climate change and at the same time some species, among which *V. unguiculata*, have shown promising healthy properties that would make them pivotal crops to address both the nutritional and the environmental security (Singh, 2020). Some of these aspects are going to be deeply investigated within this PhD thesis work.

5. African Leafy vegetables: an extraordinary source of secondary compounds and microelements

Not only legumes but also all the other plant-derived dietary patterns have been found to be below the minimum recommended intake almost worldwide. Among these, also vegetables could contribute to improve healthy diets. There are a lot of leafy vegetables neglected in the majority of the continents and only in the Middle East and North Africa they reach an adequate level of consumption to satisfy human nutritional requirements (Willett et al., 2019). Neglecting the usage of vegetables in the diet means to lose the uptake of a wide range of compounds whose provision is mandatory to deal with different nutritional related disorders, such as micronutrient deficiencies or oxidative stress damages (Sivakumar et al., 2018). Also in this case, the exploration of biodiversity in a typical bioprospecting approach may be useful to identify suitable species to fill up these nutritional deficiencies.

Leafy vegetables are considered among the best sources of anti-oxidant and anti-inflammatory compounds such as carotenoids and phenolics whose biosynthesis is functional to defend plants from the negative effects of UV radiations (Del Valle et al., 2020) and in the case of carotenoids is complementary to the photosynthetic activity (Hashimoto et al., 2016). At the same time, leaves are great sources of minerals and vitamins. These compounds are essential to be assumed in the diet since their biosynthesis does not occur in animal tissues and primary producers (i.e., plants) are a fundamental source of them (Hounsome et al., 2008). In Africa, indigenous and traditional leafy vegetables have been used as key ingredients of local diets and contribute substantially to nutrition and food security among the poor and smallholder farmers in rural and urban/peri-urban areas (Neugart al.,

2017). The nutritional components of AIVs include ascorbic acid, β-carotene, calcium, copper, iron, magnesium, manganese, potassium, and zinc (Sivakumar et al., 2018).

However, the importance of these AIVs goes beyond their nutritional value as often folkloric reports suggest the presence of various medicinal properties which includes various healing capabilities such as treatment of peptic ulcers, jaundice, toothache, and intestinal helminthiasis. Further, *in vitro* studies have identified that these AIVs have antioxidant, anti-inflammatory and anticancer properties (Table 1) are linked to their bioactive metabolites content such as the presence of flavonoids, phenolic acids, glucosinolates and vitamins which have the potential to prevent NCDs (Moyo et al., 2020).

Species	**Extract**	**Concentration (μg/mL) (dw)**	**Assay**	**Cell Line**	**Cell Viability (%)**
A. cruentus	Ethanolic	6.25	MTT	HCT-116	90.76 ± 0.07
A. cruentus		800	MTT	HCT-116	25.22 ± 0.03
A. spinosus	Methanolic	< 10	MTT	HT-29	50
C. olitorius	Ethanolic	2.4 ± 0.05	MTT	LACC	50
C. olitorius		2.7 ± 0.03	MTT	LACC	50
O. gratissimum	Ethanolic	2.5 ± 0.05	MTT	LACC	50

O. gratissimum		2.6 ± 0.12	MTT	LACC	50
S. nigrum	Aqueous	948	MTT	HT-29	50
S. nigrum		541	MTT	DLD-1	50

Table 1: AIVs extracts cytotoxic activity against some common cancer cell lines of the gastro-intestinal trait. Source: Moyo et al., 2020.

Since many of these metabolites are synthetized in plants as a response to stressful conditions (Akula & Ravishankar, 2011) it is of interest to evaluate if there are suitable conditions to grow crops in order to gain a better nutritional profile, for instance by considering the antioxidant content or the provision of vitamins. By identifying species able to face the detrimental effects of drought, competition with other plant species and low nutrients availability (as in the case of African indigenous vegetables) we avoid at the same time the negative effects posed by yield loss. Defence mechanisms to counteract many different environmental stresses have been linked to the biosynthesis in plants of phenolic compounds, involved in the reduction of the oxidative damages triggered by growing in harsh conditions such as drought, saline stress, pest attack (Del Valle et al., 2020). Leafy polyphenols usually are found in the forms of glycosides, galactosides or rhamnosides and sometimes they are associated to further moyeties such as malonilic or acilic groups to be transported within plant tissues. This condition has been found to be linked to a better absorption at the gastro-intestinal level since the glycosylic bond protects them from degradation, especially in the case of

anthocyanins (Teng & Chen, 2019). During this PhD thesis work, I considered a group of these plants; however, my work focused on *Corchorus olitorius* L., also known as jute mallow, for its value in term of both nutritional and sustainability properties. The genus *Corchorus* is comprised of annual or short-lived perennial herbs and shrubs with many agriculturally useful species. It is widely known for its high genetic diversity and geographical distribution and *C. olitorius* is from Africa (Nguomo et al., 2017). The vegetable is extensively grown for the sliminess of the leaves used in local dishes. It is made into a common mucilaginous soup or sauce in some African cooking traditions, therefore its consumption is commonly performed after cooking. It is one of the leading leafy vegetables in West Africa (Ola et al., 2009). Its leaves are collected from May to December and used also as a potherb. *C. olitorius* has also been largely produced in arid-region of Middle East and Africa, where it is used as an important vegetable for common cooking. *C. olitorius* has also revealed tolerance response to soil moisture and salt stress. It is hypothesized that the distribution of *C. olitorius* in arid-region is attributed to its tolerance to drought and harsh environmental conditions (Dhar et al., 2018). The leaves of this plant are rich in antioxidants, such as vitamin C, vitamin E, β-carotene, α-tocopherol, glutathione and phenols. The leaves also contain fatty acids, minerals, other vitamins and mucilaginous polysaccharides (Ismail et al., 2018). Moreover, this species has been linked to a wide panel of bioactive properties that make it of interest in the field of nutraceutics and nutritional prevention (Wadgy et al., 2019; Park et al., 2018; Handoussa et al., 2013; Oboh et al., 2012; Li et al., 2012). The aim of the present work is to understand if the phytochemical composition of *C. olitorius* leaves may be ameliorated by certain agronomic conditions such as those promoted by conservation agriculture and to assess putative bioactive properties

directly related to the metabolic variations triggered by the environmental growth conditions.

6. Take home messages

Many evidences suggest that there is an urgent need to develop new strategies to make food provision for mankind less impacting in terms of natural resource exploitation as also suggested by the EU Green deal guidelines (https://ec.europa.eu/info/sites/info/files/european-green-deal-communication_en.pdf). This has been further highlighted by the outbreak of the present COVID-19 pandemic, whose origin is probably linked to the Sars-COV-2 spill-over from wildlife animals to humans in contexts where the naturality of the habitats (which forecasts different ecological niches for these two categories) is not preserved (Galimberti et al., 2020). If this pandemic will teach us something, it is that the environment is not a passive system and that humans cannot overwhelm nature. It is even more necessary to start from biodiversity, for example by learning about the relationship between the environment and plants, to respectfully exploit it to obtain essential nutrients for human well-being. At the beginning of this PhD project the theme was to identify plant species and agricultural systems to reduce human impacts on the agronomic environment. Today, the main question is to ascertain which sustainable strategies and technological innovation are the most promising to satisfy the global population food demand, avoiding the resources collapse and preventing new pandemic risks. Moreover, the stakeholders involved in the food supply chain, need to focus on guaranteeing the provision of healthy foods. In this context, the identification and the encouragement to discover and adopt new crops perfectly fit this kind of necessity.

Overall, my PhD project aims at evaluating the feasibility of adopting an integrative approach to link food security and environmental sustainability in order to outline

a new food system capable of responding to the needs of the modern citizen and environmental conditions. In the light of the above-mentioned issues, three main consideration can be stated:

(i) Food safety is a global issue. The unsafety of local food markets, like the Wuhan's one and the lack of balance between anthropized and natural environments can exert severe impact at the global health level.

(ii) Local biodiversity and agrobiodiversity play a key role in 'feeding' cities, by helping to maintain resilient, equitable, culturally appropriate food systems and to promote sustainable diets.

(iii) Acknowledgment that urban and peri-urban agriculture and the conservation agricultural strategy may offer opportunities to protect and integrate biodiversity into urban landscapes and food systems, thereby contributing to synergies across food security, ecosystem services, and human wellbeing.

7. Aim of the PhD project

The general aim of this PhD project is to study local plant biodiversity (i.e., minor crops and proto-domestic species) to redesign the agriculture of the future according to the principles of nutritional quality and agricultural sustainability. Technically, I identified some African minor crop species suitable to support the SDGs promoted by the UN Agenda 2030, with particular concern for SDG n.2 ("Zero hunger") which is focused on the sustainability of food production in order to end hunger and to promote low impacting agriculture.

To go towards the goals proposed by the SDG n.2, it is essential to reduce agrochemicals, mechanization and water demand in the agriculture sector. For this reason, plant species able to grow in conservation agriculture conditions were selected. Another key issue concerns dietary healthy aspect: in this context I especially focused on the micronutrients and bioactive compounds contents of the selected plant species. The goal is not limited to studying the effects provided by single compounds but to extend this kind of investigation at the phytocomplex level. The aim is to identify plants rich in compounds able to prevent the outbreak of NCDs such as tumors or aging related diseases.

The first practical aim of this work is to identify, within the African plant biodiversity, the most resistant crops able to counteract the stressful conditions posed by the environment, such as drought and low agrochemical inputs, without affecting yield and at the same time able to maintain (or better, to improve) their nutritional value. To achieve this goal, an assessment of African plant species in urban and peri-urban areas of the city of Arusha in Tanzania was performed. Once identified the species of greatest interest for their nutritional values, I carried out

an experimentation under controlled conditions to evaluate the effect of no-tillage and water deficiency on the chosen plants. This evaluation is not only focused on plant yields: a particular attention is destined to the analysis of plants healthy properties, due the synthesis of bioactive compounds, to promote human wellbeing and prevent (instead of cure) the outbreak of chronic diseases. The selection of the studied species was made at first by evaluating a wide panel of traditional vegetables common in households and small holdings for their nutritional properties, then we focused on two of them basing on their genotypes. In particular, we aimed at focusing on species of clear African genotypes (i.e., originating in the African continent) while avoiding genera that, despite their diffusion in African countries, originated in other continents, such as the case of Amaranthus and Solanum genera.

The first crop studied is *Vigna unguiculata* (L.) Walp.. This is a member of Fabaceae and was selected due to its ability to grow in harsh environments, as well as to its interesting nutritional features and bioactive compounds content.

The response of this species to the conservation agriculture management compared to the conventional one is considered in terms of both morphological features and nutritional parameters of the main consumed portions (i.e., the seed). The evaluation of the nutraceutical properties of *V. unguiculata* seeds is widely deepen, both at the cancer preventive and aging delaying level.

The second investigated species is *Corchorus olitorius* L.*,* a leafy vegetable. This species was selected because usually this kind of vegetables (e.g., spinach) is highly water demanding and vulnerable to environmental stressors. However, African environmental conditions have shaped this plant at the genetic and phenotypical level to make it much more tolerant to abiotic and biotic stresses. Therefore, I

decided to analyze the response of *C. olitorius* to the same conservative conditions in terms of yield and phytochemicals. In particular, we addressed our research to understand how the conservation management (compared to the conventional one) could trigger the biosynthesis of specific bioactive secondary metabolites such as antioxidant compounds.

Simultaneously to these investigations, I focused on the bioactivity aspects of these two species paying attention at both individual phytochemicals and at the whole phytocomplex. In this context, I focused on developing both a chemical analysis approach for plant extracts and a methodology able to estimate bioactivity responses at the cellular level.

The general objective is to direct the cultivation and processing strategies of raw materials (e.g. cooking) to optimize the content of beneficial active ingredients.

Overall, the obtained results allowed to support the adoption of both the species in the context of conservation agriculture practices to reduce the impact of the intensive agriculture systems. These evidences are further supported by the healthy properties highlighted by the bioactivity analysis. These features are due to the occurrence of specific compounds mainly produced as a defense against biotic and abiotic stressors. The themes addressed in the introduction (Chapters 2, 3, 4 and 5) have been valorized by the publication on international journals of scientific papers entirely reported in the next chapters. Moreover, in the appendix, I annex 6 further works which I was involved in supplementing my PhD work in the field of environmental sustainability and particularly concerning by products valorization, plant-pollinators interactions, floral mimicry, DNA barcoding exploitation for food purposes and grape-wine microbial quality influencing variables.

8. Experimental design

The experimental design followed within this PhD work is summarized in Fig. 6.

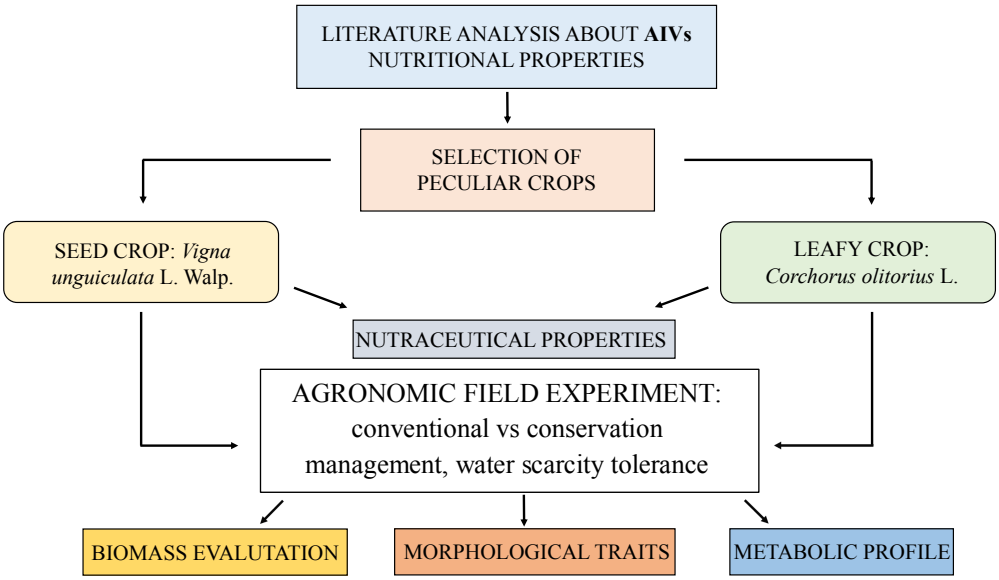

Fig. 6: experimental design followed within the present PhD thesis.

The first step of the work was a detailed literature evaluation and field survey in Arusha (Tanzania) of the environmental and nutritional properties of some AIVs. This evaluation led to the selection of two AIVs to be used in the next experimental steps due to their environmental and nutritional properties.

The first one, *Vigna unguiculata* (L.) Walp., also known as cowpea, belongs to the family Fabaceae and it is of interest mainly for the bean, even if in Africa also leaves are consumed in traditional meals. The second species selected is *Corchorus olitorius* L., commonly named as jute mallow. It is a leafy crop belonging to the family Malvaceae and consumed for the nutritional value of its leaves.

The first year of my PhD was dedicated to field experiments conducted both in Africa and in Italy. Specifically, the agronomic field experiments were held in San Bonico (Piacenza) at the Cerzoo fields where both the selected species were grown under different agronomic conditions (Fig. 7): conventional agriculture management (i.e., tillage, absence of cover crops) and conservation agriculture management (i.e., no tillage, cover crop maintenance). In both cases the cultivation was conducted in different plots treated with normal and reduced irrigation.

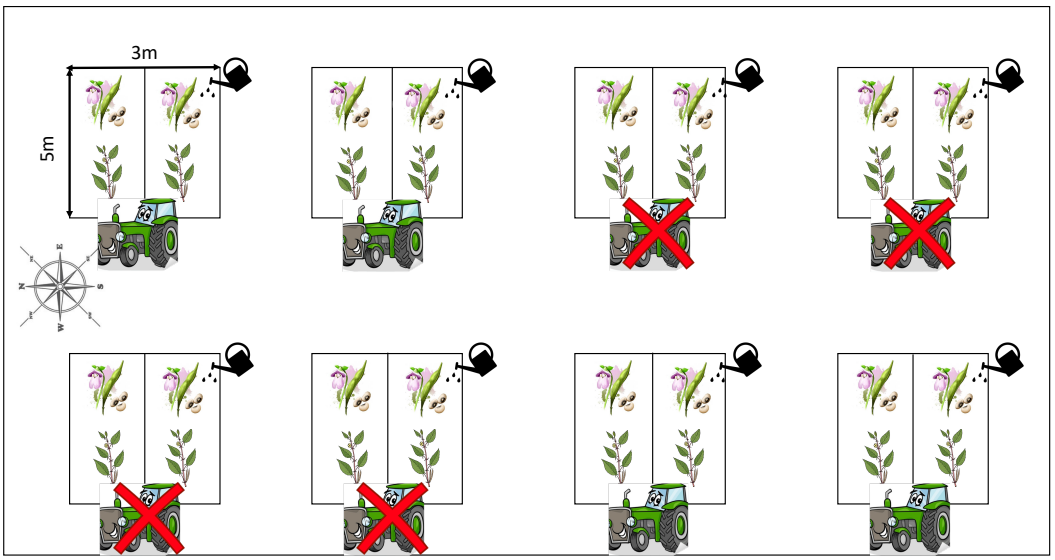

Fig. 7: Experimental design of the agronomic field experiment. Four tilled plots and four no-tilled plots were seeded with *V. unguiculata* and *C. olitorius* plants. Each plot was subdivided in two subplots. The southern sublopts were normally irrigated, while the northern were subjected to water exhaustion.

The agronomic experiment lasted 4 months, during which morphological growth traits were registered and were followed by the evaluation of the total biomass of

both crops and the harvesting of their edible portions: *V. unguiculata* seeds and *C. olitorius* leaves. These portions were subjected to phytochemical investigation to assess their metabolic profile and eventual variations triggered by the growth conditions, with particular concerns compounds of nutritional interest.

To carry out these evaluations, the experimental design involved the development of extraction systems able to retrieve target components. I tried to preserve the original conditions of consumption as much as possible in order to assess putative bioactive effects in the light of the condition of consumption of the tested species. The extracts were subjected to both target and untarget chemical analyses to identify the bioactive components of interest. Bioactivity tests were then set up using different cell models. Most of the studies focused on healthy and cancer human cells of the colon epithelium since the gut is certainly one of the target organs where bioactive foods can act.

A second activity concerned the assessment of metabolic aging induced by *V. unguiculata* phytocomplexes of the two plants. In this case, yeast cells were initially used as model and subsequently the results were also confirmed on *in vivo* models. All these analyses were performed starting from the bioactivity displayed by the whole phytocomplex and then specific fractions were studied to identify the putative bioactive compound(s).

9. Publications produced within this PhD thesis work

1. Ausilia Campanaro, Nicola Tommasi, Lorenzo Guzzetti, Andrea Galimberti, Ilaria Bruni, Massimo Labra (2019). DNA barcoding to promote social awareness and identity of neglected and underutilized species having valuable nutritional properties. *Food Res Int.*, *115*, 1-9.

In this work it is performed a systematic review about the nutritional value of AIVs, with a particular concern for micronutrients and bioactive compounds composition. Since in many countries of the Sub-Saharan Africa, AIVs are considered marginal species and neglected because of the spread of staple crops, a comparison between the nutritional value of each AIV with the staple reference is performed for at least two categories of nutritional compounds. Finally, to promote the increasing of the knowledge of such AIVs, the technique of the DNA barcoding is suggested to implement the identification of AIVs at the species level for multiple purposes, for instance as a preparatory tool for breeding programs.

General considerations: During the experimental activities in Africa I was allowed to ascertain the cultivation of numerous AIVs in the open field and to confirm many of the considerations answered in this review. Finally, this first review work was fundamental for the selection of the two species tested in the PhD project.

Type of Article: Mini-Review Article

DNA barcoding to promote social awareness and identity of neglected, underutilized plant species having valuable nutritional properties

Running title: NUS distribution and authentication

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Abstract

It is estimated that about 7,000 plant species and a large number of cultivars and varieties have been cultivated for consumption in human history. However, < 0.5% of these currently provide the majority of human food energy needs worldwide (e.g., rice, wheat, maize, and potato). Global issues such as climate change, diffusion of pests, and resistance to agrochemical treatments are posing great concern about the sustainable cultivation of these major staples, especially in equatorial and tropical countries, such as Sub Saharan Africa. In addition, most of these regions contain malnutrition and micronutrient deficiencies, and the sum of such problems create serious implications at social, political, and economic levels. A possible solution relies on the exploitation of plant biodiversity and particularly on the so-called NUS (Neglected and Underutilized Species). These plants are traditionally grown in their centres of origin and continue to be maintained by sociocultural preferences, however they remain inadequately documented and neglected by formal research and conservation programs. Although they are important in terms of micronutrients and the ability to grow in harsh conditions, these species are falling into disuse due to agronomic, genetic, economic, and cultural reasons.

To promote and spread their cultivation at the global scale, along with knowledge on their suitability for human nutrition, reliable identification systems are necessary to guarantee adequate authenticity along the entire supply chain and distribution network. A precise identification of the different species and their varieties is fundamental both to retrieve information on their origin and authenticate the raw materials (i.e., seeds, leaves and fruit) and related processed products that can be distributed at the local or global scale. DNA-based techniques can help achieve this

mission. In particular, the DNA barcoding approach has gained a role of primary importance due to its universality and versatility. Here, we discuss the advantages in using DNA barcoding for the identification of some of the most representative NUS species, as well as their traceability and conservation of cultural practices around them.

Keywords

DNA barcoding, Food Security, Micronutrient deficiency, NUS, Sub Saharan Africa.

1. Introduction

1.1 From plant domestication to the opportunity and critical issues of the Green Revolution

The domestication of wild plants has been a pivotal step in human history that determined the shift from hunter–gatherers to agricultural societies and stimulated the rise of cities and modern civilization. Humans still rely on the same crops that were domesticated around 10,000 years ago in different regions of the world such as Central America, New Guinea, and the Fertile Crescent (Purugganan & Fuller, 2009). From these areas, most of the cultivated plants spread worldwide due to the progressive globalization process (Zeder, 2008). Despite the relevance of agriculture, two-thirds of global plant-derived food is provided by only three major crops – maize (*Zea mays*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*). Our dependence on such crops limits our capability to deal with challenges posed by the adverse effects of climate change and the consequences of dietary imbalance (Cheng, Mayes, Dalle, Demissew, & Massawe, 2017).

From the 1960s through the 1980s, the so called Green Revolution focused on improving agronomic and nutritional features of the major staple crops (e.g., maize, rice and wheat) diffused during primary and secondary domestication processes. The principal goals were the enhancement of plant productivity and increasing the content of macronutrients, such as carbohydrates and proteins (Evenson & Gollin, 2003). Most of the plant varieties selected during the Green Revolution were also methodically bred to deal with emerging environmental and biotic stresses, and they were expected to produce yields several times higher than local cultivars.

Unfortunately, the problems of modern agriculture remained largely unresolved. In particular, the diversity of crops and their varieties is decreasing, and the general trend of farming intensification led to the concentration of pests, thus causing a general loss of productivity for the target species of the Green Revolution (Pingali, 2012). Given these conditions, farmers are more and more asked to select new crop varieties providing enhanced yields and resistance to pathogens and environmental stresses, such as desertification. A putative solution to these problems could be represented by the introduction of Genetically Modified Organisms GMO. However, this approach could not be economically sustainable in those areas characterized by high levels of poverty (Bazuin, Azadi, & Witlox, 2011). A more effective approach, in terms of both the economic and environmental sustainability, is represented by the adoption of ecological intensification practices (Kovács-Hostyánszki et al., 2017). In this framework it could be possible to enhance crop yields by supporting for example pollinators' density and diversity and to tackle pests and pathogens occurrences by sustaining the communities of their natural enemies (Garibaldi et al., 2016). Furthermore, the adoption of plant species and varieties well-adapted to water scarcity could act as a reliable strategy in conditions of desertification (Mabhaudhi, Chimonyo, Chibarabada, & Modi 2017).

The first category of problems affecting modern agriculture are those related to climate change (Zilberman, Lipper, McCarthy, & Gordon, 2018). With the average global temperatures predicted to increase above 3.0°C by midcentury, agricultural productivity is expected to significantly decline in many countries (Abraham et al., 2014) as has already been demonstrated for many important staples, notably maize, wheat, and rice, due to their sensitivity to water shortages and heat stress (Wheeler & Von Braun, 2013; Khoury et al., 2014). Additionally, the lack of diversity

within gene pools in modern crops reduces the possibility of producing new resistant genotypes and leaves the current agricultural systems vulnerable to the global issues mentioned above (Fuller et al., 2018; Zilberman, Lipper, McCarthy, & Gordon, 2018). A second issue of global concern in agriculture is related to the nutritional properties of the cultivated species and their effect on human health. Major crops for example are unbalanced in terms of carbohydrates vs. micronutrients (Burchi, Fanzo, & Frison, 2011). An opportunity comes from those regions not interested by the Green Revolution, or at least where it did not impact consistently. For example, Sub-Saharan Africa experienced a lag in the benefits provided by the modern crop varieties of Green Revolution due to the political and pedoclimatic conditions (Ejeta., 2010). Therefore, there are many autochthonous edible plants that could be seen as unexplored items in the context of agrobiodiversity and that could contribute to improve agricultural issues worldwide. For example, some legumes such as the pigeon pea *Cajanus cajan* are rich in proteins, carbohydrates and micronutrients, and due to their limited spread are more resistant to environmental stresses such as pests, scarcity of water and fertilization (Odeny et al., 2007; Saxena, Kumar, & Sultana, 2010). Similarly, some green leafy vegetables such as *Amaranthus* spp. and *Solanum* spp. (e.g, *S. aethiopicum*, *S. macrocarpon*, *S. melongena* and *S. scabrum*) are well diffused in Sub Saharan countries and are more resistant to climatic stress than the well-known and largely cultivated common spinach (*Spinacia oleracea*). These species are also richer in micronutrients such as vitamins and minerals (Chivenge, Mabhaudhi, Modi, & Mafongoya, 2015). Although their agronomical and nutritional potential, the main problem of such local African species is related to their current use and diffusion which are often scarce and limited to local markets or even to achieve

subsistence at the family level. For these reasons, such plants have been generally referred to as Neglected Underutilized Species (NUS). The most common definition of NUS refers to those crops excluded from the scientific research, from the improvements of the Green Revolution and from the main distribution chain (Hammer, Heller, & Engels, 2001; Padulosi, Thompson, & Rudebjer, 2013; Naveena, Mouzam, & Bellundagi, 2016). Nowadays, the term NUS could also be extended to every alternative use of a crop or their portions. This latter point refers for example to the cowpea (i.e. *Vigna unguiculata*) which seeds are usually the target of consumption, but in Southern African countries its leaves are also eaten cooked or dried (Ahenkora, Dapaal, & Agyemang, 1998).

The main reasons that limit the expansion of these crops concern agronomic, genetic and economic issues (Padulosi et al., 2013; Njeru, 2016). Regarding the first two aspects, the NUS can be considered as proto-domestic species, since they have not been selected during the centuries on the basis of their higher productivity or seeds dimension, as in the case of major staple crops (Snir et al., 2015). Moreover, NUS often lack a complete food supply chain supporting their commercialization (Will, 2008). Multidisciplinary studies and programs linking informatics, genetic analysis, and traditional knowledge are increasing the awareness and the access to NUS (Padulosi, Bergamini, & Lawrence, 2012; Dansi et al., 2012). In some cases, innovative farming strategies of NUS and dedicated breeding programs increase the diffusion and the market value of these plants (Neugart, Baldermann, Ngwene, Wesonga, & Schreiner, 2017); however, in most cases NUS and knowledge around them are scarce, partial or progressively disappearing (Mellisse, Descheemaeker, Giller, Abebe, & van de Ven, 2018).

To preserve NUS biodiversity and enhance their utilization, it is necessary to stimulate the dialogue among farmers, scientists and final consumers through a participatory approach aimed at improving smallholder agroecosystems, diets, livelihoods, and global food security (Afari-Sefa et al., 2016). Enhancing the nutritional and agronomical positive features of NUS and implementing their use will hopefully lead to the creation of a supply chain that supports the commercialization of such species (Mabhaudhi et al., 2017). This scenario requires a precise knowledge of the involved species and of their traditional uses. However, in many cases, each crop identified by a common name (e.g., amaranth or nightshade) could encompass an astounding list of varieties and different species or hybrids. It is therefore essential to rely on efficient analytical systems to provide a unique identification of NUS plants and related food items.

1.2 Discovering the NUS

The term NUS refers to a large panel of species including legumes, grains, fruit, and vegetables adapted to a wide panel of environmental conditions mainly related to drought and soils poor in organic matter and micronutrients. The higher resilience of NUS to harsher conditions than the major cereal crops, make them suitable to grow in low-input farming systems in marginal environments and to face the environmental stress conditions posed by climatic change issues. Many African NUS such as *Cleome gynandra* and *Amaranthus* spp. are C4-photosynthetic species (Marshall et al., 2007; Tsutsumi, Tohya, Nakashima, & Ueno, 2017) therefore they can save more water than thee common C3-based crops (Griffiths, Weller, Toy, & Dennis, 2013). For these reasons, plants like the quinoa (*Chenopodium quinoa*),

once considered NUS, have recently received research attention and dedicated breeding programs to enhance their quality and productivity features (Chivenge et al., 2015), since they are more resistant than other staple crops to water scarcity (Jacobsen, 2003). Furthermore, NUS are suitable for intecropping with staple crops, enhancing crop productivity and nutrient-use efficiency and providing at the same time the basis for a better balanced diet (Ebert, 2014).

Another important characteristic of such plants refers to their higher nutritional properties in terms of macro and micronutrients content with respect to those of common staple crops (Padulosi, Bergamini, & Lawrence, 2012; Nyadanu & Lowor, 2015). Micronutrients deficiency is a globally shared problem as in the case of iron, vitamin A, and zinc. Iron deficiency in developed countries is treated by introducing more meat and some iron-rich vegetables (e.g., *S. oleracea*) into the diet. Interestingly, some NUS, such as *Corchorus olitorius* and *Vernonia amygdalina* are richer in iron than common staple crops such as spinach (Table 1). Concerning vitamin A, the global deficiency in children of pre-school age is about 25% (FAO, 2017) and the main source of this vitamin in staple crops is represented by potatoes and carrots. However, some dark leafy green vegetables from the Sub Saharan areas such as *Corchorus olitorius* and *Cleome gynandra* (Table 1) are richer in this and other vitamins rather than globally diffused staples.

Other important compounds of nutraceutical interest are amminoacids, which are highly represented in many NUS legumes, as *Vigna subterranea*, *Vigna unguiculata* and are two times higher than the common bean *Phaseolus vulgaris* (Table 1).

Concerning plant secondary metabolites, given their importance in the modern diet for health and wellness, it is important to underline that a diet rich in traditional leafy vegetables such as spider plant, jute mallow and amaranths could promote

the uptake of many healthy-promoting compounds, such as glucosinolates, polyphenols and betalains. Glucosinolates are secondary compounds typical of the Brassicales showing great anti-inflammatory and antioxidant activities. They are 10 times more concentrated in *Cleome gynandra* than in the staple crop *Brassica oleracea* (Table 1). Polyphenols are compounds widely spread in the plant kingdom but they are of high-added value for their nutraceutical and pharmacological interest. Leaves of traditional NUS such as *Corchorus olitorius* or V*ernonia hymenolepis* are richer in polyphenols than the common staple *Spinacia oleracea* (Table 1). Betalains are secondary compounds of interest for their anti-inflammatory activities and are typical of the Caryophyllales. In amaranths, they are more concentrated than the common staple *Chenopodium quinoa* (Table 1).

Species	Origin	Common name	Edible portion	Most abundant micronutrients per 100g	Principal metabolites per 100 g	Comparison with representative staple crops	References
Amaranthus sp	America	Amaranth	Leaves boiled	Ascorbic acid 126 mg; Ca 425 mg; Mg 224 mg FW	Betalains 7.73 mg FW	Ascorbic acid > 2 times *Citrus x sinensis*, Ca 2 ~ times *Brassica oleracea*, Mg > 2.5 times *Spinacia oleracea*, betalains higher than quinoa	Kruger, Sayed, Laghenhoven, & Holing, 1998; van Wick & Gericke, 2000; Steyn, Olivier, Winter, Burger, & Nesamvuni, 2001; Maughan et al., 2012;

						(0.15 - 6.1 mg/100g)	Biswas, Dey, & Sen, 2015; Abderrahim et al., 2015
Cleome gynandra	sub-Saharan Africa, Asia	Spider plant	All young plant's part coocked as vegetables (the leaves may also be dried after coocking)	β-carotene 3 g; Fe 2.9 mg; Folate 346 µg FW	Glucosinolates 294 mg FW	β-carotene > 100 times *Ipomoea batatas*, Folates ~ 2 times *Lens culinaris*, glucosinolates > 10 times *Brassica rapa* (20 mg/100g)	Kruger et al., 1998; van Wick & Gericke, 2000; Chen, Zhu, Gerendás, & Zimmermann, 2008; Stoessel, Juraske, Pfister, & Hellweg, 2012; Onyango,

							Kunyanga, Ontita, Narla, & Kimenju, 2013; Omondi et al., 2017; Wu, Solberg, Yndgaard, & Chou, 2018
Corchorus olitorus	Africa	Jute mallow	Leaves (coocked) eaten with porridge	Ascorbic acid 105 mg, β-carotene 5 g, Ca 265 mg, Fe 20-54 mg, Folates 100 µg, Zn 5 mg FW	Polyphenols 0.25 g FW	Ascorbic acid 1.39 times, Ca, Fe > 3 times *S. oleracea*; polyphenols > 1.5 *S. oleracea* (0.15 g/100g)	van Wick & Gericke, 2000; Roy et al., 2006; Hanif, Iqbal, Iqbal, Hanif, & Rasheed, 2006; Stoessel et al.,

							2012; Ko et al., 2014; Nyanadu & Lowor, 2015; Giro & Ferrante, 2016
Galinsoga parviflora	South America	Gallant Soldier	Leaves	Ca 162 mg, Mg 681 mg, Mn 44 mg FW	Polyphenols 3.5 g DW	β-carotene > 200 times *I. batatas*, Mg 10 times *S. oleracea*; polyphenols higher than *S. oleracea*	Udosen, Udok, & Unuigbe, 1999; van Wick & Gericke, 2000; Odhav, Beekrum, Akula, & Baijnath, 2007; Ranilla, Kwon, Apostolidi, &

							Shetty, 2010; Ko et al., 2014
Justicia flava	Africa	Yellow justicia	Leaves and preboiled young seeds	Ca 2073 mg, Fe 16 mg, Zn 11 mg DW	Lignans (amount not available)	Incredible source of Ca: more than 20 times higher than in *S. oleracea*	Olaniyi & Powell, 1980; van Wick & Gericke, 2000; Hanif et al., 2006; Odhav et al., 2007
Lablab purpureus	India	Lablab	Leaves and preboiled young seeds	α-tocopherol 2.77 mg, Ca 58.5 mg, Fe 6.78 mg, Zn 2.66 mg, FW	Aminoacids 18.25 g DW	Total tocopherol content higher than cereals, quinoa and common bean,	van Wick & Gericke, 2000; Rychlik, Englert, Kapfer, & Kirchhoff, 2007; Habib, Al

						Zn 2 times *Phaseolus vulgaris*	Meqbali, Kamal, Souka, & Ibrahim, 2014
Lasianthera africana	Africa	-	Leaves	Ca 800 mg, Fe 77 mg, Zn 36 mg FW	Polyphenols 1.821 g DW	Ca ~ 10 times, Fe > 3 times *S. oleracea*	Udosen et al., 1999; van Wick & Gericke, 2000; Hanif et al., 2006; Atiko, Onocha, & Oyedemi, 2016
Launaea cornuta	Africa	Bitter lettuce	Leaves	Ascorbic acid 15.9 mg, Ca 265 mg, Fe 2,7 mg FW	Many antimalarial compounds (e.g. polyphenols,	Ca 3 times *S. oleracea*	van Wick & Gericke, 2000; Lyimo, Temu, & Mugula, 2003;

					amounts not available)		Hanif et al., 2006
Senna occidentalis	South America	Coffee senna	Seeds and leaves	Ca 513 mg, Fe 3 mg, Zn 2 mg FW	Amminoacids 62.13 g PE	Amminoacids and Zn 2 times *P. vulgaris*	van Wick & Gericke, 2000; Henderson, 2001; Odhav et al., 2007; Rychlik et al., 2007; Oshoke & Akyniemi, 2015
Solanum aethiopicum, nigrum, scabrum,	South America	Nightshade	Leaves, fruit	Ascorbic acid 75 µg, β-carotene 5.8 µg, Ca 194 mg,	Polyphenols 42.1 g DW	Ca > 2.5 times; polyphenols ~ 300 times *S. oleracea* (0.15 mg/100g)	van Wick & Gericke, 2000; Hanif et al., 2006; Ko et al.,

macrocarpon, dulcamara				Fe 3 mg, Folates 70 µg FW			2014; Ronoh et al., 2017
Tylosema esculentum	South Africa	Marama bean	Seeds	α-tocopherol 2.88 - 3.77 mg, Ca 93.7 - 217.6 mg FW	Phenolic acids and flavonoids 406.6 mg FW	Superior in composition to soya beans	van Wick & Gericke, 2000; Holse, Husted, & Hansen, 2010; Chingwaru, Vidmar, Kapewangolo, Mazimba, & Jackson, 2015
Vernonia amygdalina	Africa	Sweet bitterleaf	Leaves	Ascorbic acid 46.64 mg, β-carotene 9.05	Flavonoids 11.5 g DW	Ca, Fe and flavonoids	van Wick & Gericke, 2000; Bergquist,

				mg, Ca 1264.1 mg, Fe 32.2 mg DW		higher than *S. oleracea*	Gertsson, Knuthsen, & Ollson, 2005; Hanif et al., 2006; Usunobun & Okolie, 2015
Vigna subterranea	Africa	Bambara groundnut	Seeds	Ca 220 mg, Fe 7 mg, Zn 8 mg FW	Amminoacids 68.6-76.6 g PE	Amminoacids > 2 times, Zn 8 times *P. vulgaris*	van Wick & Gericke, 2000; Massawe, Dickinson, Roberts, & Azam-Ali, 2002; Olaleke, Olorunfemi, &

							Akintayo, 2006; Rychlik et al., 2007
Vigna unguiculata	Africa	Cowpea	Seeds, leaves	Ca 66.4 mg, Fe 6.7 mg, Folate 149-152 µg, Mg 54.6 mg, Zn 6 mg FW	Amminoacids 63.9 g PE	Amminoacids > 2 times, Zn 6 times *P. vulgaris* (16), Folate 1.5-15 times *Pisum sativum* (10-87 µg/100g)	Olaleke et al., 2006; Rychlik et al., 2007

Table 1 - Examples of NUS providing high contents of micronutrients and secondary compounds and comparison with values of common and widespread staple crops. To provide an immediate comparison, only the maximum concentration of each micronutrient/secondary compound is reported. (FW = fresh weight, DW = dry weight, PE = protein extract).

NUS represent an important opportunity not only for their nutritional properties, but also for local socio-economic development. Many local NUS have the potential for being transformed in novel food products ready for distribution and are able to generate economic incomes for communities of smallholder farmers (Gruère, Giuliani, & Smale, 2006; Kahane, 2013). The introduction of NUS also constitutes a way to reduce the risk of over-reliance on very limited numbers of major crops and a way to increase sustainability of agriculture through a reduction in inputs, such as fossil fuel-derived and nitrogen fertilizers, given the high impact of the carbon footprint of agriculture on climate change (Will, 2008; Ebert, 2014).

International bodies, such as the World Vegetable Center (https://avrdc.org/) have the mission of finding and safeguarding many NUS and the cultural aspects related to their cultivation and use. For example, this center is working actively to improve *Amaranthus* ssp. and *Vigna subterranea*, and also distribute seeds to local farmers. Although well distributed locally, the same plants could be considered NUS at the global level and their poor marketability and limited access make them largely underutilised in both social and economic terms (Padulosi & Hoeschle-Zeledon, 2004). The worldwide agricultural sector needs to recognize the importance of NUS to enhance food security issues (Mayes et al., 2011) and to guarantee the transmission of traditional knowledge about their uses to the future generations.
The first step to valorize the NUS at the global level and enhance their diffusion is the development of a suitable species identification system, as well as the identification of their nutritional features. These plants are generally proto-domestic or spontaneous in local contexts and it happens that the same species could be known under different common names. This is the case for example of

spider plant and African cabbage which refers to the same NUS, *Cleome gynandra* (Omondi et al., 2017). Conversely, different species can be grouped under the same common name such as *Amaranthus viridis* and *A. dubius.* that are usually referred to as Amaranth.

Among the available tools for species identification and traceability, DNA barcoding is the most universal, cheap, and inclined to automatization and rapid application as supported by a huge number of studies (see for example Hollingsworth et al., 2011; Galimberti et al., 2014; Mishra et al., 2016). Therefore, in this review we intend to remark the advantages of universal molecular tools (such as the DNA barcodes), in promoting some species from a neglected and underutilized situation to a relevant role in modern agriculture and to authenticate their seeds and related food items. This could represent one of the main concrete actions to support Sub Saharan NUS as an element of global importance for a more sustainable and healthy diet.

1. DNA barcoding to address NUS authentication

In the last decades, DNA-based methods have been used to certify and trace several cultivated plants, such as rice, corn, barley, rye, and grape. Since 2003, a universal method known as DNA barcoding has been progressively diffusing to authenticate wild and cultivated species (Casiraghi, Labra, Ferri, Galimberti, & De Mattia, 2010; Galimberti et al., 2013). This is a simple and cost-effective (<1$ per sample, Meier, Wong, Srivathsan, & Foo, 2016) tool based on the characterization of universal DNA regions able to unequivocally identify organisms at the species level. As a general

principle, ideal DNA barcode regions should have a high interspecific and low intraspecific variability. However, in some cases (e.g., De Mattia et al., 2011; Enan, & Ahmedet, 2014) this approach also allowed to characterize cultivated species at deeper taxonomic levels such as subspecies and cultivar.

In the case of metazoans, the ideal barcode region is a 658 bp portion of the mitochondrial COI (Casiraghi, et al., 2010), while in plants the choice of the best barcode region/s was motivated by the guidelines of the International DNA Barcoding Plant Working Group (IDBPWG), that encouraged the use of the plastidial *rbcL* and *matK* regions (Hollingsworth et al., 2009). Furthermore, the plastidial intergenic spacer *trnH-psbA* and the nuclear ITS region were progressively adopted due to their higher variability (that permits the differentiation among congenerics) and the already documented success in identifying herbal and traditional medicine products (De Mattia et al., 2011; Galimberti et al., 2014; Mezzasalma et al., 2017).

Although the molecular approach at the basis of DNA barcoding is not new to science, the strength of this method relies on the availability of reference sequences, belonging to morphologically recognized voucher specimens, that are archived by international platforms such as the BOLD (Barcode of life database) system. Reference barcode sequences are indeed the key stone for a correct identification and the starting point for the discovery of new entities (Casiraghi et al., 2010). BOLD is a public repository supporting the collection of DNA barcodes, with the aim of creating a reference library for all living species (Ratnasingham, & Hebert, 2007). BOLD is used to assign a given DNA barcode to both a vouchered specimen, validated by expert taxonomists, and other DNA barcode sequences belonging to the same or different taxa. The lack of reference sequences is the main limit of the method. While some group of organisms are well represented in such

databases, a lot of work is still necessary to provide a reliable source of reference DNA barcodes for groups which have been poorly investigated. Given the need for reference DNA barcodes to improve the reliability of the identification and the lower public resonance concerning NUS, we advocate for the creation of a dedicated NUS dataset that could serve as both a reference for identification and a repository for the conservation of these species. However, nowadays, many reference sequences for the barcode regions ITS2, *matK* and *rbcL*, necessary for NUS identification, are available in public international databases such as GenBank-NCBI (https://www.ncbi.nlm.nih.gov/nuccore), as reported in Table 2. Looking at the species listed in this table, it is possible to find some examples of possible application of DNA barcoding in the case of NUS. In many cases, given subtle morphological similarities, it could be difficult to discriminate among species belonging to the same genus such as in *Amaranthus* (Achigan-Dako et al 2014), or even among species belonging to different genera when the edible portions of the plant have been processed. This is case for example of *Launaea cornuta,* which can be easily replaced by *Sonchus oleraceus* or *Launaea taraxacifolia* (Denton, Schippers, & Oyen et al 2004). A similar risk regards the genus *Senna* for which many different species could be adopted to prepare traditional health remedies. Seethapathy and colleagues (2015), reported the possibility and the need to distinguish *Senna* species through the DNA barcoding approach, since market analysis revealed frequent adulteration events (Seethepathy et al., 2015).

Preventing adulteration is of particular interest especially for processed NUS products such as dried leaves or flour. This is the case of *Vigna unguiculata* and *Cleome gynandra*, which are traditionally dried for conservation, or the *Vigna subterranea* which is often stored as flour. In small and medium farms of

developing countries, adulteration could also derive from accidental substitution operated by seed distributors (even including governmental bodies, NGOs, small companies) and not from intentional frauds due to economic advantage. As stated by Barriga and Fiala (2018), recent efforts by governments and donors to address the problem of seed quality has been tending to focus on cer- tification and labeling and to reduce the possibility of adulteration by downstream sellers (see also Louwaars & De Boef, 2012). Such products are morphologically unidentifiable and only a DNA-based approach could better clarify the identity of the original raw material. A similar situation recently occurred in the field of traditional (or neglected) medicinal plants, where remarkable progresses have been made through the use of DNA barcoding to promote their exploitation and the globalization of their market (see for example the reviews by Techen, Parveen, Pan, & Khan, 2014; de Boer, Ichim, & Newmaster, 2015; Raclariu, Heinrich, Ichim, & de Boer, 2018). To date, most NUS lack of reference DNA barcoding accessions such as *Vernonia amygdalina*, *Justicia flava* and *Tylosema esculentum* and probably for many other NUS not mentioned in this study. This underlines the need to encourage the production and submission of reference accessions of NUS to enhance the adoption of the DNA barcoding as a standard tool to support NUS traceability along the entire supply-chain. An efficient molecular identification approach would enhance not only food safety issues but also it will support the regulation of the exploitation local genetic resources. In this context, the Nagoya protocol, adopted on 2010 by the contracting states to the United Nations Convention on Biological Diversity (CBD), represents a legal instrument on the subject of accessing to genetic resource and the fair and equitable sharing of benefits arising from their utilization

(Myburgh, 2011). DNA barcoding could represent a valid tool to support the adoption of Nagoya protocol, especially in developing countries that are more and more interested in safeguarding their biodiversity and valorize traditions and usages about NUS and other local genetic sources.

NUS	Distribution	Topic of Investigation	Reference DNA barcodes	References
Amaranthus spp.	worldwide	Species authentication	a) ITS2 - b) rbcL - matK	a) Kuzmina et al., 2017; b) Burgess et al., 2011
Cleome sp.	worldwide	Biogeography	a) ITS2 - b) rbcL	a) Feodorova, Voznesenskaya, Edwards, & Roalson, 2010; b) Cardinal-McTeague, Sytsma, & Hall, 2016
Corchorus olitorius	worldwide - tropical	Genetic evolution - Species authentication	a) ITS2 - b) rbcL - matK	a) Benor, Fuchs, & Blattner, 2011; b) Xu et al., 2018
Galinsoga parviflora	worldwide	Phylogeny - Species authentication	a) ITS2 - matK - b) rbcL	a) Blöch et al., 2009; b) Xu et al., 2018

Justicia flava	Africa	Taxonomy	ITS2	Kiel, Daniel, Darbyshire, & McDade, 2017
Lablab purpureus	worldwide - tropical	Species authentication - Phylogeny	a) ITS2 - rbcL b) matK	a) Kuzmina et al., 2017; b) Stefanović et al., 2009
Lasianthera africana	Africa	Species authentication	matK - rbcL	Parmentier et al., 2013
Launaea cornuta	Africa	Phylogeny	ITS2 - matK	Kim, Chunghee, & Mejías, 2007
Senna occidentalis	worldwide - tropical	Species authentication - Adulteration product	a) ITS2 - b) matK - rbcL	a) Mao, Xia, He, Liu, Y., Liu, F., Zhao, & Liang, 2017; b)

				Seethapathy et al., 2015
Solanum aethiopicum, nigrum, scabrum, macrocarpum, dulcamara	worldwide	Phylogeny - DNA barcoding	a) ITS2 b) rbcL - matK	a) Särkinen et al., 2015 b) Group et al., 2011
Tylosema esculentum	Africa	Plant physiology	rbcL	Mitchell, Keys, Madgwick, Parry, & Lawlor, 2005
Vernonia amygdalina	Africa	n.a	n.a	n.a
Vigna subterranea	Africa	Genetic diversity - Phylogeny	a) ITS2 - rbcL b) matK	a) Takahashi et al., 2016; b) Wojciechowski, Lavin, & Sanderson, 2004

Vigna unguiculata	worldwide - tropical	Genetic diversity - Phylogeny	a) ITS2 - b) matK - c) rbcL	a) De Luca, Cennamo, Del Guacchio, Di Novella, & Caputo, 2018; b) Wojciechowski et al., 2004; c) Stefanović et al., 2009

Table 2 DNA barcoding characterization of NUS. For each species or genus, the distribution, the availability of published DNA barcoding studies and of reference barcode sequences in the international GenBank is reported.

On the whole, we can consider DNA barcoding as a valuable tool to valorize NUS for three main aspects.

-Knowledge diffusion and transfer.

Most NUS species are cultivated and used at the local scale, sometimes only by small communities inhabiting a certain geographic region or even by groups of families due to ancient cultural transmission episodes. In other cases, their use is much more common, but they are known under different trivial names, thus impeding a reliable diffusion and conservation effort. A suitable DNA-based identification approach allows to reach standardization and thus to develop a system able to unambiguously identify the genetic resources and assign them to a certain name (Galimberti et al., 2014). Moreover, any deposited reference barcode sequence could be integrated with information on provenance and features such as productivity, water, and soil requirement and the resistance to pests and environmental stress conditions. Such data could improve the worldwide distribution of NUS and a dedicated DNA barcoding database for those species could also have a great impact on the agronomic and scientific community. An established species (and varieties) database is indeed the first step to stimulate research programs on plants genetics and physiology to improve crop yield in different climatic regions.

- *Standardization of identification processes.*

DNA barcoding has rapidly affirmed its efficacy among different categories of stakeholders and is nowadays a gold standard for the global food market. In fact, it was proposed by the US Food and Drug Administration for the authentication of

food items such as the seafood (Sheata, Naaum, Garduno, & Hanner, 2018). Similarly, DNA barcoding could play a key role in verifying the identity, mislabeling and safety of herbal products used as food or food supplements (Raynor, Dickinson, Knapp, Long, & Nicolson, 2011). We expect that in the next future this approach will be widely applied to several food items, also in response to the adoption of the Nagoya Protocol. In this framework, a universal and standardized system with great economic and political resonance could certainly be adopted to certify NUS and even their varieties.

- *Universality of the method.*

The universality of the DNA barcoding allows the application of similar protocols for the identification of different matrices, significantly simplifying the analytical approach to food analysis. Furthermore, this method could facilitate the access of smart analytical systems to all the supply chain steps up to the final consumer. For example, many studies coupled the universality of DNA barcodes with the sensitivity of High Resolution Melting (HRM) to authenticate plant products (see for example Madesis, Ganopoulos, Anagnostis, & Tsaftaris, 2012 and Sun, Li, Xiong, Zhao, & Chen, 2016). More recently, Valentini and co-workers (2017) demonstrated that the precision of DNA barcoding can be easily coupled with the high sensitivity of nanotechnologies to provide rapid and user-friendly colorimetric systems to achieve food authentication (Valentini et al., 2017). These kinds of innovations will make molecular-based identification affordable to non-specialized personnel, also in those regions where high-tech laboratory facilities are not available. In particular, in contexts where NUS are getting more diffused, these types of innovation are pivotal to ensure food safety in the supply chain and to avoid adulteration events.

2. Conclusions

It is clear that a sustainable future of agriculture is realizable only by exploring biodiversity at the species and variety levels. Crop genetic diversity is only a key dimension of overall biodiversity. The re-discovery of NUS represents a pivotal element to introduce new plants for cultivation at the global scale. We can consider this strategy a sort of modern green revolution that starts from plant biodiversity to improve farming systems but also considers the cultural, social, political, and economic issues of each interested region. Although these species have seeds, leaves or fruits rich in micronutrients, some of them could resemble other taxa having indigestible parts or secondary metabolites that could be not suitable to human nutrition. Such food safety issues can nowadays take advantage of reliable identification systems like the DNA barcoding one, allowing to assess NUS biodiversity and their distribution. This possibility ensures protection to consumers, producers, and all the stakeholders involved in the NUS supply chain against adulteration and species substitution events. At the same time, the DNA barcoding authentication of NUS contributes to preserve their biodiversity, ethnobotanical knowledge and agricultural sustainability by enhancing farm-level resilience to ongoing global environmental phenomena, such as climate change.

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2. Lorenzo Guzzetti, Andrea Fiorini, Davide Panzeri, Nicola Tommasi, Fabrizio Grassi, Eren Taskin, Chiara Misci, Edoardo Puglisi, Vincenzo Tabaglio, Andrea Galimberti, Massimo Labra (2020). Sustainability perspectives of *Vigna unguiculata* L. Walp. cultivation under no tillage and water stress conditions. *Plants*, *9*(1), 1-15.

This work concerns the evaluation of the suitability of *V. unguiculata* (commonly named as cowpea) to conservation agriculture management (i.e., no tillage and cover crops maintenance) and to low hydric regimes. Growing conditions between conservation and conventional management are monitored and with them also the response to hydric stress is assessed. The parameters evaluated range from morphological traits to biomass and yield evaluation and take also into account some metabolic features of seeds produced by plants under different treatments, with particular concerns for compounds of nutritional interest (i.e., starch, proteins, amino-acids).

General considerations: The results of this work confirmed what was qualitatively observed during African inspections. The added value of this work was that for the first time it was confirmed that this plant is also well suited to the Mediterranean environment in conditions of conservation agriculture.

Sustainability Perspectives of *Vigna unguiculata* L. Walp. Cultivation under No Tillage and Water Stress Conditions

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Abstract: Nowadays, agriculture is facing the great challenge of climate change which puts the productivity of the crops in peril due to unpredictable rain patterns and water shortages, especially in the developing world. Besides productivity, nutritional values of the yields of these crops may also be affected, especially under low mechanization and the low water availability conditions of the developing world. Conservation agriculture (CA) is a topic of emerging interest due

to the provision of adequate yields and reduced environmental impact, such as greenhouse gas emissions, by being based on three main principles: minimum soil disturbance (reduced or no tillage), cover crop maintenance, and crop rotation. The aim of this study was to assess the impact of CA management on the growth performance and the nutritional profile of cowpea (*Vigna unguiculata* L. Walp), a pulse of African origin, commonly known as black eye bean under field conditions. A field experiment was designed to assess the effect of conventional tillage (CT) and no-tillage (NT) combined with the usage of a set of cover crops, coupled to normal and deficient water regimes. Cowpea was revealed to be able to grow and yield comparably at each level of the treatment tested, with a better ability to face water exhaustion under CA management. After a faster initial growth phase in CT plots, the level of adaptability of this legume to NT was such that growth performances improved significantly with respect to CT plots. The flowering rate was higher and earlier in CT conditions, while in NT it was slower but longer-lasting. The leafy photosynthetic rate and the nutritional profile of beans were slightly influenced by tillage management: only total starch content was negatively affected in NT and watered plots while proteins and aminoacids did not show any significant variation. Furthermore, significantly higher carbon and nitrogen concentration occurred in NT soils especially at the topmost (0–5 cm) soil horizon. These findings confirm the capability of CA to enrich soil superficial horizons and highlight that cowpea is a suitable crop to be grown under sustainable CA management. This practice could be pivotal to preserve soils and to save agronomical costs without losing a panel of nutrients that are important to the human diet. Due to its great protein and aminoacidic composition, *V. unguiculata* is a good candidate for further cultivation in regions of the word facing deficiencies

in the intake of such nutrients, such as the Mediterranean basins and Sub-Saharan countries.

Keywords: conservation agriculture; no-tillage; climate change; drought stress

1. Introduction

The Green Revolution (1960–1980s) was aimed at improving the agronomic productivity and nutritional features of the major staple crops worldwide (e.g., maize, rice, and wheat) [1]. Most of the crop varieties were selected to deal with emerging environmental and biotic stresses (i.e., desertification, nutrient-poor soils, and extreme temperatures) and were expected to produce yields several times higher than minor crops and the local varieties. Unfortunately, overcoming some of these obstacles was not always possible in a sustainable way and during the past three decades, cultivation practices have been demanding a higher and higher use of water and agrochemicals (e.g. fertilizers, pesticides, and herbicides), to enhance (or maintain) maximum crop yields [2,3]. Environmental hazards, the poor maintenance of long-term plant and soil productivity and the higher costs in terms of agrochemicals and energy consumption produced the modern crisis of agriculture. To address this crisis and environmental concerns of the consumers, in recent years, the principles of Sustainable Agriculture were continuously promoted worldwide [4,5]. Therefore, for the green revolution of the 21st century, the practices of Organic farming (OF) and Conservation Agriculture (CA) are deemed environmentally friendly approaches to agriculture. Traditionally, OF is based on the creation of the correct ecosystems for the crop productivity with a holistic approach that considers maintenance and health of the soil, plants, and livestock,

with strictly regulated use of external inputs while focusing on farm production and recycling of needed products (e.g., composting wastes and green mulching) and the adoption of integrated strategies against plant pests. On the other hand, CA represents a set of three crop management principles: (i) direct planting of crops with minimum soil disturbance, (ii) permanent soil cover by crop residues and cover crops, (iii) crop rotation [6]. Through these strategies, CA guarantees an optimum environment for the rhizosphere to capture nutrients and water [5]. The adoption of no-till (NT) and the maintenance of a crop residue mulch on the surface have assumed an important role, especially in the geographical areas characterized by consistent rainfall and the consequent risk of soil leaching [7]. Although most of the production zones in the Mediterranean region are characterized by hot summers and rainy winters, global warming has been increasing the risk of (i) soil degradation due to soil losses in response to the greater drought and torrential rainfall; (ii) soil salinization due the increase of droughts, irrigation, and sea level; and (iii) soil carbon stock depletion because of the increase of temperature and drought [8]. Therefore, the application of CA principles has the potential in the Mediterranean regions to preserve soil structure and fertility, as well as to improve productivity and quality of crops [9–11].

Promotion and research on CA in many instances have focused on the first two principles, which are minimum soil disturbance/no-tillage and surface crop residue. Species belonging to Fabaceae could also enhance soil fertility thanks to the nitrogen-fixing symbionts. Moreover, there are several legume crops that are also able to grow under stress conditions [12], such as water/salt stress, and could be adapted to no-tilled soils. Sub-Saharan Africa is an important source of stress-resistant legume grain cultivars, such as species belonging to the genera *Vigna* and

Lablab [13–15], and some of these could also be adapted to grow under the Mediterranean climatic conditions.

In this study, we selected *Vigna unguiculata* L. Walp (also known as cowpea) to investigate the ability of this species to grow in the Mediterranean region under CA conditions. The species was adequate for such study due to the fact that its beans are rich in proteins and carbohydrates and have relatively low-fat content [16]. Moreover, *Vigna unguiculata* beans show an aminoacidic pattern that is complementary to that of many foods consumed in the Mediterranean area, such as cereal grains. These aspects make *V. unguiculata* a 'strategic' crop for the Mediterranean diet. Furthermore, *V. unguiculata* is attracting the attention of consumers and researchers due to its beneficial health properties, including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory, and anti-hypertensive properties [17].

Specifically, in this work, the response of *V. unguiculata* to NT soil management both with and without irrigation was investigated. Plant growth features and plant productivity, in terms of straw biomass and grain yield, were evaluated. At the same time, the response of the soil carbon (C) and nitrogen (N) stock to NT was verified. Emissions of greenhouse gases CO_2 and N_2O from NT soils are highly variable and depend on complex interactions among soil properties (i.e., soil water content, soil C and N), microbes, and the cultivated plant. Usually, the increased soil organic C (SOC) in surface layers of no-till soils is widely found but may not be associated with increased C sequestration throughout the soil profile [18]. Therefore, the evaluation of the relative carbon balance under NT vs. CT is essential to better estimate the potential of NT to sequester additional C into the soil. Furthermore,

there is no accordance in the scientific literature about the effect of NT in N sequestration and dynamics [18].

Moreover, the metabolic features of the seeds, in terms of nutritional components after boiling (to imitate the conditions of consumption and the effective intake for humans), were assessed.

2. Material and methods

2.1. Experimental design and treatments

A one-year field experiment was carried out on a long-term field study (initiated in 2010) at the CERZOO experimental research station in Piacenza (45°00'18.0" N, 9°42'12.7" E; 68 m above sea level), Po valley, Northern Italy. The soil is a fine, mixed, mesic, Udertic Haplustalf (Soil Survey Staff 2014), with a silty clay loam texture (sand 122, silt 462, and clay 416 g kg^{-1}) in the upper layer (0–30 cm). The main physical-chemical properties of the soil are reported in Fiorini et al. [19]. The climate is temperate, and the mean annual temperature and precipitation are 12.2 °C and 890 mm, respectively. Climatic data were collected from an automated meteorological station positioned in the experimental field (Figure S1 in Supplementary Material). The experimental design was a randomized complete block (RCB) with four repetitions and two tillage treatments: conventional tillage (CT) and no-tillage (NT). In detail, (i) CT included an autumn plowing (35 cm) and two passages of rotating harrow in spring (15–20 cm) to prepare the seedbed, and (ii) NT consisted of direct sowing on a soil untilled for 7 years using a double-disk opener planter for seed deposition. Between 2011 and 2017, the crop sequence was a three-year crop rotation, with soybean (*Glycine max* L. Merr.), durum winter

wheat (*Triticum turgidum* L. var. durum), and maize (*Zea mays* L.). During winter off-seasons, a mixture of winter cover crops was sown in NT plots, right after harvesting the previous main crop. The species composing the cover crops mixture were rye (*Secale cereale* L.), hairy vetch (*Vicia villosa* L.), crimson clover (*Trifolium incarnatum* L.), Italian rye-grass (*Lolium multiflorum* Lam.), and radish (*Raphanus sativus* L.). In 2018, 15 m^2 (5 m × 3 m) within each plot (1430 m^2; 65 m × 22 m) were cropped with *V. unguiculata*, both under CT and NT. The experiment was established to compare responses of cowpea cultivation to contrasting tillage systems. NT and CT planters were calibrated in order to obtain the same sowing depth in both treatments. The distance between planting rows was 50 cm, and the distance between seeds on the same row was 10 cm. Sowing of cowpea was carried out on May 18th (sowing depth: 3–4 cm) and the harvest took place on August 9th. No fertilizers were applied during the growing season, and weeds were suppressed weekly by hands.

On July 20th, when cowpea plants were at the beginning of the blooming, each plot was divided into two subplots. The first one was sprinkler irrigated to prevent water stress (20 mm per time, for a total of three irrigation events), whereas, in the second sub-plot, any kind of natural or artificial water input was prevented by temporarily covering those sub-plots through greenhouses to induce and to simulate the dry season.

2.2. Biomass and morphophysiological sampling and analysis

During the whole growing seasons, plants (N = 320) were measured weekly for a total of 7 surveys to detect the following parameters: the dimension of the canopy (cm), the total number of leaves, and number of flowers. Plants were labelled

univocally in order to follow the growing performance of each individual over time. After the greenhouses settlement, to evaluate the role of water exhaustion on plants, in each subplot three plants were randomly chosen to be undergone to Pocket PEA Chlorophyll Fluorimeter (Hansatech Instruments, Pocket PEA, 2013) measurements, providing an estimate of the F_v/F_m ratio. F_v is the fluorescence variable, calculated as F_m - F_o, where F_o is the fluorescence origin and F_m is the fluorescence maximum.

Measurements took place from the 5th to the 7th survey on the same labelled plants in a time range between 10 am and 1 pm.

After the 7th survey, grain yield and above-ground biomass weight were measured by harvesting three randomly selected 2.0 × 1.0 m squares from each subplot. Above-ground biomass was manually cut at the soil level and weighed. Grain and straw were also separated. The dry weight biomass of cowpea (grain and straw) was gravimetrically determined by drying biomass at 70°C until constant weight.

Fruits derived from the remaining plants were harvested and stored at −20°C before phytochemical analyses.

2.3. Measurement of soil physical properties

To determine soil C and N stock, soil bulk density (BD, 0–30 cm), SOC, and N concentration in the 0–20 cm layer (0–5 and 5–15 cm) were measured right after harvesting cowpea. Four randomly selected undisturbed soil core samples were collected on August 20th, 2018 from each subplot, using a steel auger of 5 cm diameter. Soil BD was determined according to the cylinder method [20], while samples to determine SOC and N concentration were air-dried, ground with a

rubber pestle, and sieved to 2 mm. About 1 g of dry soil per each sample was weighed and used to determine C and N concentration by Dumas combustion method with an elemental analyzer varioMax C:N (VarioMax C:NS, Elementar, Germany). Soil carbonate removal was not necessary due to the low carbonate content in the soil.

To estimate the effect of watering on soil, the gravimetric water content was measured on a weekly basis, both in watered (W) a non-watered (NW) plots (Figure 1). From May 18th to July 20th precipitation events occurred for a total of 121 mm. After July 20th, in W sub-plots, precipitation and irrigation events consisted of 38 and 60 mm (three irrigations of 20 mm each), respectively.

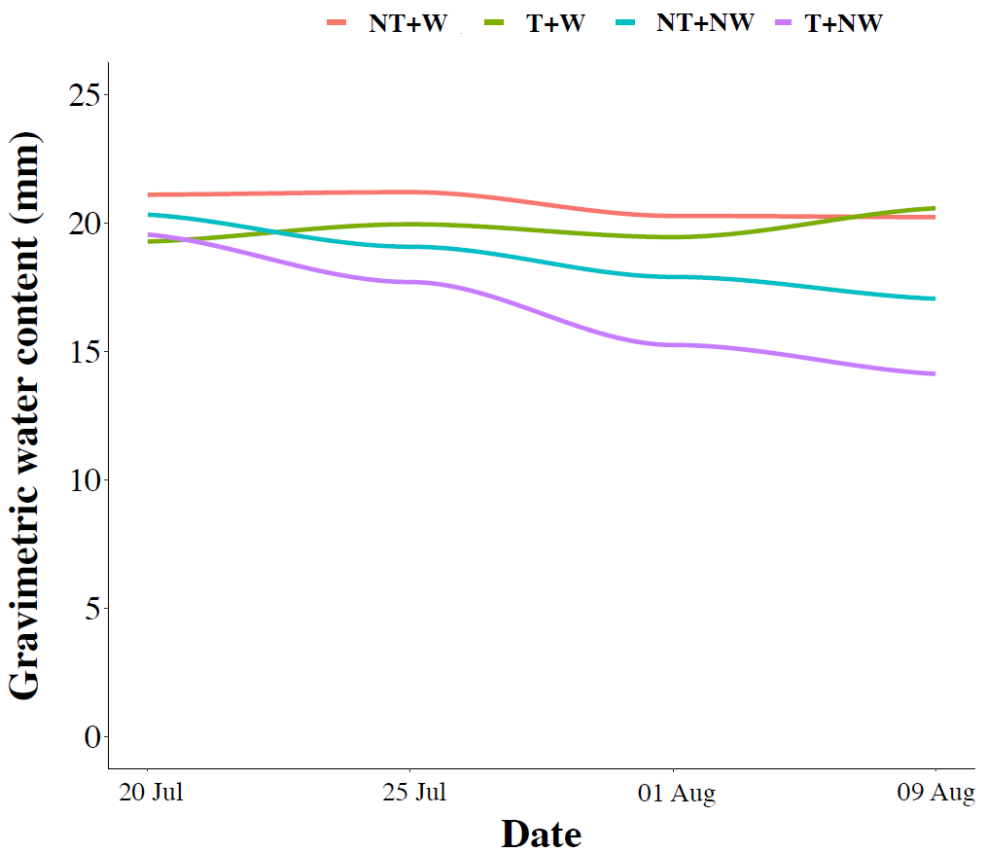

Figure 1. Trend of the gravimetric water content in the four different treatments. CT = conventional tillage, NT = no tillage, W = watered, NW = not watered.

2.4. Chemical characterization of V. unguiculata seeds

Phytochemical analysis was carried out on the cowpea seeds that were boiled in water for one hour and then were left to cool down for another hour, as suggested by Olaleke et al. [21], in order to mimic the conditions of cooking and consumption. Subsequently, seeds were dried at 50 °C overnight and then ground to a fine powder.

2.4.1. Evaluation of Total Starch Content (TSC)

The TSC content was indirectly evaluated by measuring the amount of NADPH in samples after an enzymatic treatment by using the Kit Megazyme® Total Starch AOAC Method 996.1 1 and AACC Method 76.13. Briefly, 50 mg of dry powder was added to 200 µL of ethanol 80% v/v and 1 mL of KOH 2M and left stirring for 20 min at 4°C. Then, 4 mL of a sodium acetate buffer 1.2 M Ph = 3.8 were added followed by the addition of 50 µL of α-amylase (8300 U/mL) and then 50 µL of amyloglucosidase (AMG, 3300 U/mL). Samples were incubated for half an hour at 50°C and then centrifuged at 3000 rpm for 10 minutes to recover the supernatant. For each sample, the reaction mixture was prepared as follows in a quartz cell: 1 mL H_2O, 25 µL of the supernatant, 50 µL of a buffer solution pH = 7.6, 50 µL NADP+/ATP. The solution was incubated for 3 min at room temperature and then the absorbance was read at 340 nm against the blank containing water instead of sample.

Then, 10 µL of a solution containing hexokinase (HK) and glucose-6-phosphate-dehydrogenase (G6PDH) was added. After an incubation of 5 min at room temperature, the absorbance was read against the blank again at 340 nm. TSC is expressed as g of total starch per 100 g of dry powder.

2.4.2. Total Protein Content (TPC)

The extraction of amino acids and proteins from dry seeds was performed as in Olaleke et al. [21] with minor modifications. Briefly, 2 g of dry powder were extracted in 50 mL of an aqueous solution of theanine at a concentration of 10 µg/mL. Theanine was chosen as internal standard as it is not biosynthesized in beans. The solution was stirred at 500 rpm for 5 minutes. Then samples were centrifuged at 5000 rpm for 30 minutes and the supernatant was recovered and freeze-dried. Yields of extraction were recorded by weighing freeze-dried extracts.

The Total Protein Content (TPC) was evaluated by using the Bradford assay as follows: 1 mL of 50% Coomassie-Brilliant Blue Bradford reagent (ThermoFisher) was incubated at room temperature with 2 µL of extract of known concentration for a minute. Absorbance was read against blank at 595 nm and fitted on a calibration curve made up with BSA (Bovine Serum Albumin) in a range between 0 and 6 mg/mL. TPC was expressed as g total proteins per g of extract and was then multiplied per the yield of extraction to be expressed on g of dry powder.

2.4.3. Amino acidic content and characterization

The evaluation of the amino acid content was performed by High Performance Liquid Chromatography coupled to a Diode Array Detector (HPLC-DAD), 1260 Infinity II LC System (Agilent, 2018). A calibration curve was made up by using an amino acid mixed solution (Merck, analytical standard, 17 amino acids plus

tryptophan) in a range between 0.078 mM and 1.25 mM. The column used for this analysis is an Agilent Poroshell HPH C18 (100 × 4.6 mm, 2.7 μm) with a guard column (AdvanceBio Oligo 4.6 × 5 mm, 2.7 μm) and it was kept at 40°C. Mobile phases were phosphate buffer (10 mM Na_2HPO_4 pH = 8.2) and Acetonitrile:Methanol:Water (45:45:10). The elution program is (%B): 0–0.35 min 2%, 13.4 min 57%, 13.5 min 100%, 15.7 min 100%, 15.8 min 2%, 18 min end. Flow rate was constant at 1.5 mL/min. Solvents were HPLC grade, whereas the buffer, solutions and samples were pre-filtered with a 0.22 μm filter. As derivatizing agent, OPA (o-Phthaldialdehyde reagent, Merck) was chosen for its capacity to bind amino groups and act as fluorophore. The injection volume was 10 μL. The signal used to visualize the fluorescence was set at 338 nm bandwidth 10 nm with a reference wavelength of 390 nm bandwidth 20 nm. Spectra were collected during analysis in a range between 200 nm and 500 nm with a step of 2 nm, to have a side control and make identifications easier. All data were displayed and analysed on Agilent ChemStation software.

2.5. Plant morphometry, photosynthetic efficiency, and phytochemistry

Data deriving from the field activity were analyzed through the software R(Version 3.3.3 © 2019–2016) and particularly by the lme4 and glmmTMB, mgcv and gamm4 package. Linear Mixed Effect Models (LME) or Generalized Linear Mixed Effect Models (GLMM) were initially applied.

However, when considering trends in time of morphological parameters, model validation confirmed non-linear pattern in the residuals, therefore, the application of GAMMs (Generalized Additive Mixed Models) was required after confirming through the AIC evaluation.

Specifically, canopy was assumed to be Gamma distributed (data were considered Gamma-distributed because of the occurrence of negative fitted values), number of leaves and flowers was considered Poisson distributed but they were then switched to a negative binomial distribution to deal with overdispersion. Concerning the evaluation of F_v/F_m ratio from PEA measurements, only data from the 5th to 7th survey were provided, so the (b) model was directly run. This ratio is an index, therefore it was assumed to be beta-distributed. Models were run providing for a double random effect, which is the individual nested within the plot. Model validation was performed by plotting residuals from each model against fitted values as well as each covariate and random component. Because of the high tendency of violation of independence as a consequence of temporal correlations (data were collected week by week), a corARMA1 correction was provided for each model [22]. The selection of the best model was provided by following the Aikaike Criterion (AIC) through the anova function.

Concerning plant chemical parameters, TPC, TSC, and total amino acidic content were analyzed through a GLMM as above, considering the plot as random factor (R, Version 3.3.3 © 2019–2016 and particularly by the lme4 and glmmTMB package). Data were assumed to be binomially distributed but necessitated a switch to a beta-binomial distribution due to over-dispersion. The fixed effect was time in interaction with the management condition (tillage, irrigation) in order to evaluate their effect on response variables in time by exploiting 95% confidence bands.

2.6. Soil properties

The soil C and N stock (Mg ha^{-1}) at 0–30 cm depth was calculated as follows: profile soil stock (Mg ha^{-1}) = (soil C/100) × BD (Mg m^{-3}) × depth (m) × 10 000 (m^2 ha^{-1}). Likewise, soil N stock on the soil was determined.

Data on soil C and N (both concentration and stock), as well as on grain yield and straw biomass of cowpea, were subjected to analysis of variance (ANOVA) with a split plot design following procedures outlined by Gomez and Gomez [23] and using the "agricolae" package of RStudio 3.3.3. The main-plot factor was the tillage system (NT *vs.* CT), while the subplot factor was water management (W *vs.* NW plots). When the Shapiro–Wilk test and the Levene's test did not confirm the assumptions of ANOVA, data were log-transformed before analysis. Tukey's honestly significant difference (HSD) as a post hoc was used to test for significant differences in variables among treatments with a *p*-value of 0.05 as the threshold for statistical significance.

3. Results

3.1. Biomass and grain yield

No significant differences were detected between grain yield on plant grown under NT and CT conditions (Figure 2) and with and without irrigation. Concerning the straw biomass, significant higher production in the W plots than in the NW ones (6.78 vs. 5.18 Mg ha^{-1}; +31%) was observed. Conversely, no difference was found between CT and NT plots (Figure 2).

The interaction between the tillage system and water supply showed significant differences in cowpea straw biomass while not in the grain yield production (Figure 2). In detail, CT-W and NT-W had the highest straw biomass with

6.94 and 6.63 Mg ha^{-1}, respectively. CT-NW had the lowest straw biomass (4.22 Mg ha^{-1}), while NT-NW did not show a significant difference compared to the other conditions (Figure 2).

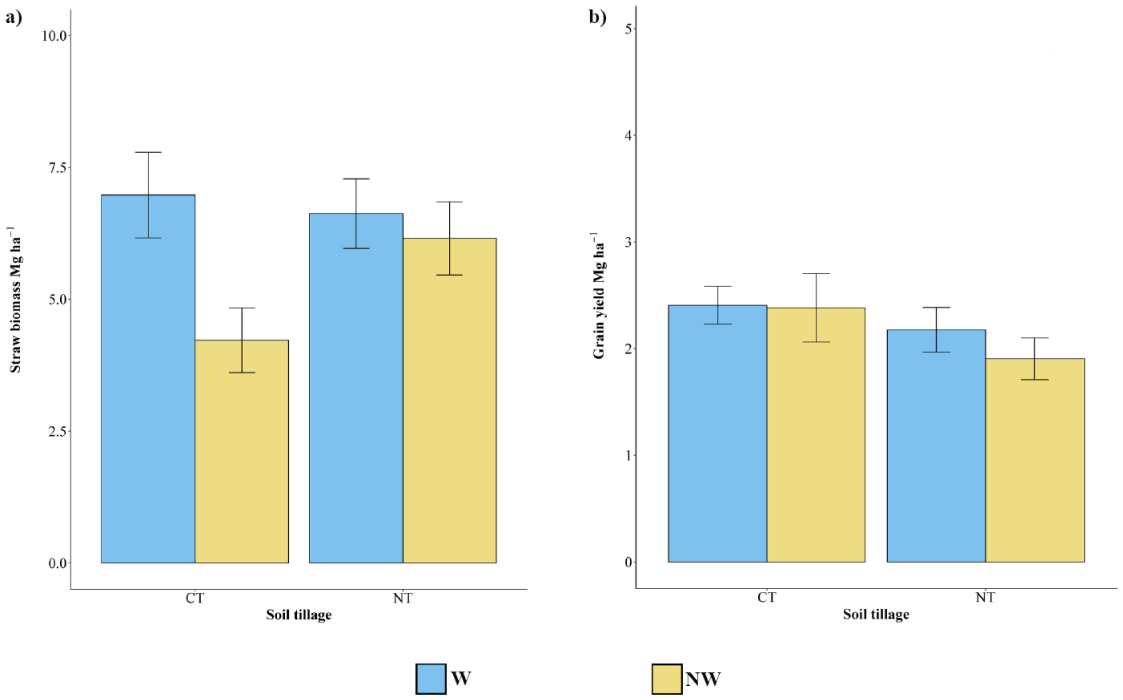

Figure 2. (a) Cowpea biomass and (b) grain yields (Mg ha^{-1}). Values are mean ± SEM. CT = conventional tillage, NT = no tillage, W = watered, NW = not watered.

3.2. Morphometrics and growth parameters

Planting density was measured in all plots and subplots at the late flowering stage (R4-R5) and was as follows: under CT, the W (CT-W) and the NW (CT-W) subplots had an average of 16.8 and 15.3 plants m^{-2}, respectively; under NT, the W (NT-W) and the NW (NT-NW) subplots had on average value of 18.5 and 17.5 plants m^{-2}, respectively.

Figure 3 shows the results related to the morphometric parameters detected: number of flowers (intended as a reproductive parameter), dimension of the canopy, and total number of leaves (intended as growth parameters). The flowering period took place starting from the 4th week. As Figure 3A shows, CT plots revealed a sudden blooming followed by a likewise sudden interruption, while NT plots showed a more contained but constant blooming. Therefore, blooming was significantly higher between the 4th and the 5th week in CT plots and then significantly higher in NT plots. No effects were due to no irrigation (see 95% confidence bands). Concerning vegetative parameters, the obtained data (Figure 3B and C) suggested that a significant difference resides in the decrease of the total number of leaves caused by the absence of irrigation at the 6th week, followed by recovery during the last survey. As far as tillage is concerned, it was found that at the beginning of the growth season (from the 1st to the 3rd week) both plants canopy expansion and total number of leaves were significantly higher in CT, then during the 4th survey, no differences were detected between the two groups, while from the 5th week to the end NT plots showed a significantly higher performance for both the considered parameters.

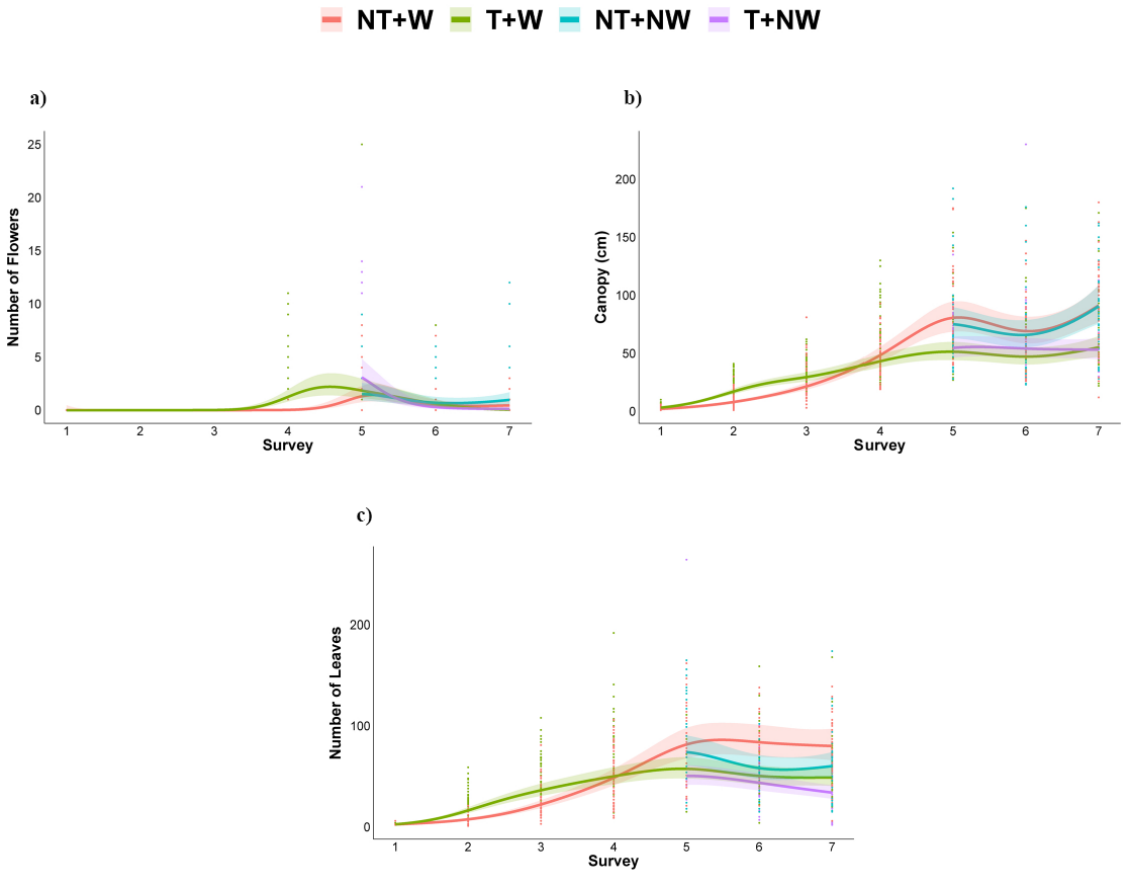

Figure 3. Models showing the trend of (a) number of flowers, (b) canopy length, and (c) number of leaves during the experimental period in the four treatments. CT=conventional tillage, NT=no tillage, W=watered, NW=not watered.

3.3. Efficiency of photosynthesis and metabolic profile

Figure 4 shows the results relative to photosynthetic efficiency, TSC, TPC, and amino acid content. Results suggested that metabolic features were lowly affected by tillage management. Photosynthetic efficiency (Figure 4) was comparable

between the two treatments (χ2 = 5.03, p = 0.17). Concerning proteins (Figure 4b), TPC was not significantly influenced by treatments (χ2 = 6.14, p = 0.11).

Also, the total amino acidic content (Figure 4d) did not show any significant difference between treatments (χ^2 = 4.15, p = 0.25), with an average amount ranging between 0.5% and 2% of the dry matrix.

Finally, TSC was clearly influenced by treatments (χ^2 = 29.63, p < 0.001). In particular, both CT (β = 0.3 ± 0.1, p = 0.004;) and NW (β = 0.29 ± 0.05, p < 0.001) caused an increase in the TSC of about 4.5% and 3.3%, respectively. The interaction resulted to be significant as NT coupled to W caused the most dramatic decrease in TSC (β = ⁻0.23 ± 0.06, p < 0.001).

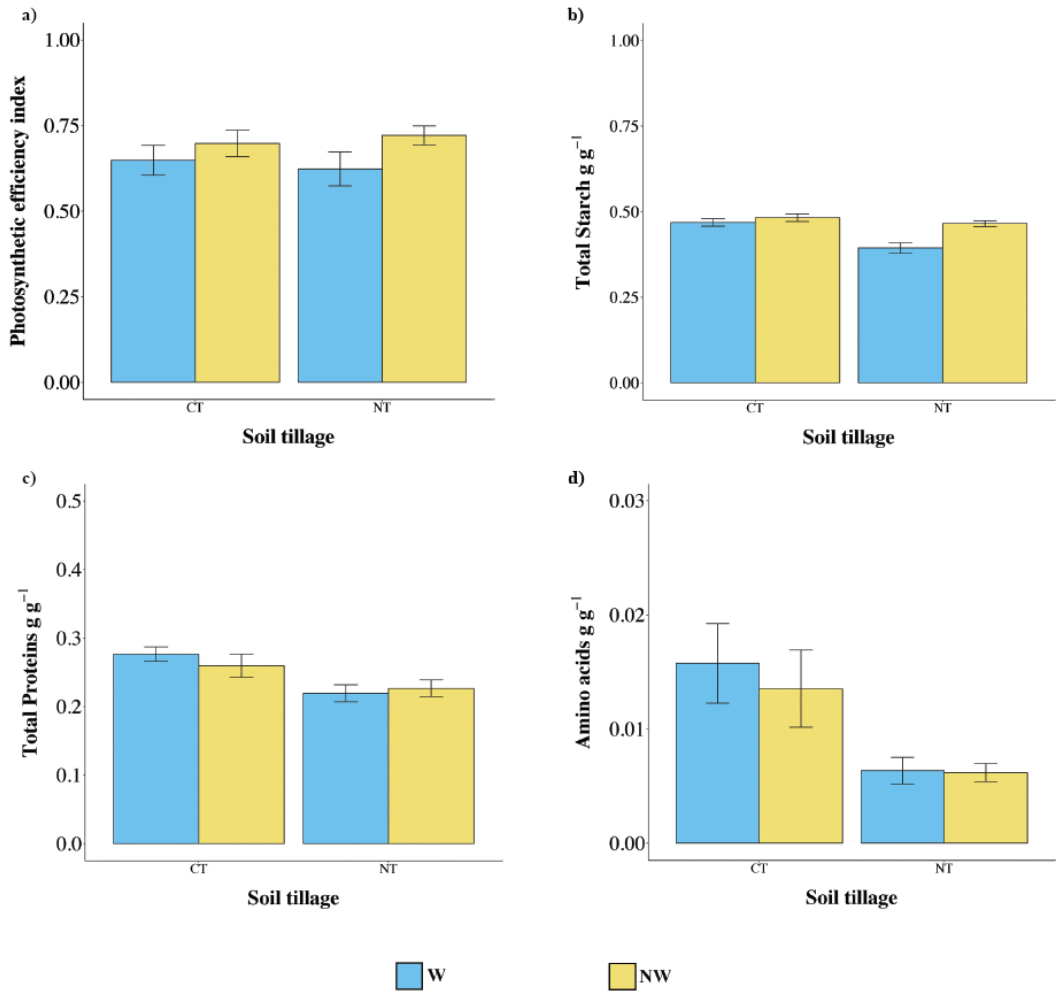

Figure 4. (a) Leafy photosynthetic efficiency, (b) TSC, (c) TPC, and (d) amino acids. Values are mean ± SEM. CT=conventional tillage, NT=no tillage, W=watered, NW=not watered.

3.4. Soil organic carbon and total nitrogen

Concerning soil chemical characteristics, a significant difference in C and N concentration occurred between NT and CT, especially at the topmost (0–5 cm) soil horizon. In detail, C and N concentrations in this soil layer were 59% and 27% higher

under NT than under CT. No significant difference in soil C and N concentration was found in the 5–30 cm soil layer. (Tables 1 and 2). Overall, soil C stock in the 0–30 cm soil layer was statistically affected by the tillage system and was 2.58 Mg ha^{-1} higher in NT than in CT soil (+5%). Conversely, soil N stock in the same soil layer did not statistically differ between NT and CT, even though NT tended to increase soil N stock value by 4% (Tables 1 and 2).

Table 1. Soil organic carbon. Values are mean ± SEM. Significance levels: * < 0.05, ** < 0.01, *** < 0.001.

Condition	Code	C concentration 0–5 cm (g C kg^{-1} soil)	C concentration 5–30 cm (g C kg^{-1} soil)	C stock (Mg ha^{-1})
Tillage	CT	12.49 ± 1.48	12.39 ± 0.88	48.56±3.37
	NT	19.92 ± 0.73	12.5±0.76	51.15±2.24
Signif.		***	n.s.	*
Water	W	16.17±4.06	12.49±0.76	49.98±2.81
	NW	16.24±4.22	12.4±0.88	49.73±3.51
Signif.		n.s.	n.s.	n.s.
Interaction	CT-W	12.46±0.92	12.43±1.03	48.69±3.53
	CT-NW	12.51±2.07	12.34±0.87	48.43±3.74
	NT-NW	19.88±0.97	12.54±0.54	51.27±1.24
	NT-NW	19.96±0.55	12.45±1.02	51.03±3.19
Signif.		n.s.	n.s.	n.s.

Table 2. Nitrogen content in the soil. Values are mean ± SEM. Significance levels: * < 0.05, ** < 0.01, *** < 0.001.

Condition	Code	N concentration 0-5 cm (g N kg^{-1} soil)	N concentration 5-30 cm (g N kg^{-1} soil)	N stock (Mg ha^{-1})
Tillage	CT	1.49 ± 0.21	1.49 ± 0.1	5.84 ± 0.46
	NT	1.9 ± 0.21	1.57 ± 0.1	6.1 ± 0.37
Signif.		**	n.s.	n.s.
Water	W	1.72 ± 0.37	1.55 ± 0.11	6.04 ± 0.39
	NW	1.67 ± 0.22	1.52 ± 0.12	5.91 ± 0.48
Signif.		n.s.	n.s.	n.s.
Interaction	CT-W	1.41 ± 0.09	1.48 ± 0.1	5.76 ± 0.37
	CT-NW	1.57 ± 0.28	1.5 ± 0.12	5.93 ± 0.58
	NT-NW	2.03 ± 0.22	1.62 ± 0.06	6.32 ± 0.08
	NT-NW	1.76 ± 0.08	1.53 ± 0.13	5.88 ± 0.43
Signif.		n.s.	n.s.	n.s.

4. Discussion

4.1. Suitability of V. unguiculata for Mediterranean CA

Our findings, overall, suggest that tilling is not fundamental to guarantee cowpea growth and yield. Therefore, cowpea is a crop suitable for CA practices and could be cultivated in harsh conditions such as arid and semiarid regions, where not all the crops perform well. For instance, grain yield in common bean *Phaseolus vulgaris* L. is highly affected (-70%) under drought conditions [24].

Usually, tillage alters the physical-chemical properties of soil, and NT vs. CT may greatly impact plant growth and yield [25]. Unfavorable effects of NT on crop yield have been widely reported immediately after the conversion from CT [26]. This is because NT may increase soil strength and BD in the initial years due to transient compaction of soil [27], thus reducing the root growth of plants [28]. However, it has also been shown that negative effect usually expires from three to five years after the conversion from CT to NT [19]. Our experimental activities with seven-year NT corroborated this improvement of yield potential under NT in the medium-long term.

No-till is usually indicated to enhance the water content of soil [29], which is of primary importance to sustain crop yield, especially under arid and semi-arid climates [30]. Our data show that NT tended to mitigate soil water losses compared with CT and cowpea tolerated NW conditions well under NT.

These results are in line with earlier studies reporting the high potential of NT to enhance crop yield in non-irrigated field management [31]. The efficacy of cowpea to grow under water deficient conditions and with reduced or no tillage was also documented by Moroke et al. [32] in the experimental area of Texas, and

by Ahamefule and Peter [33] in Nigeria. Moroke and co-workers [32] suggested that the cultivation of this species enhances the residual soil water and the presence of surface residue management due to cover crops and NT systems usually increases the whole stored soil water.

In the Mediterranean basin, mean temperatures are constantly increasing and precipitation pattern is changing towards hot and dry summer seasons as a consequence of climate change. In this context, European production of pulses has been constantly declining (from 5.8 to 1.8 million ha from 1961 to 2013), mainly due to the introduction of more specialized and intensive crops such as wheat, rice, and corn [34–36], but also as a consequence of productive instability of highly water-demanding species [37]. However, the European Commission [38,39] welcomes initiatives to increase the EU's plant protein production in a sustainable and agro-ecological way. Since cowpea can be considered as a leguminous species with reduced water demand and high drought tolerance [40], it may be considered to support the cultivation of legumes in Europe replacing currently cultivated species that have higher water demands [37]. In addition, we indicate that cowpea is also a reliable alternative to the common Mediterranean bean (*P. vulgaris*) in North-African countries, especially because of high drought tolerance during the reproductive phase [41]. Therefore, combining NT through the cultivation of species and cultivars of pulses highly resistant to water stress (such as the cowpea) could support the resumption of legume cultivation in Europe and in the Mediterranean basin to deal with the ongoing claims about pulses and climate change [42].

Concerning the growth parameters, our experiment shows that plants initially grow better on CT soils, but after having joined a critical dimension the NT

treatment helps to maintain and stimulate a more pronounced growth in a significant manner. This is particularly true if soil treatment is associated with NW. Generally, the two treatments on the global pattern are not significantly different, but this is due to a balance on the whole values associated with pretty large variations during the growing season. Also, leafy photosynthetic efficiency did not differ between treatments. Therefore, we highlight a great capability of the cowpea to grow under reduced tillage and low water regime.

Many studies focused on the impact of CA on crop yields [6], while no attention was given to morphological adaptations and metabolic profiling. The latter is an important aspect, impacting on nutritional importance and sustainability of this crop.

Generally, the main constituents of the seeds were not strongly affected by treatments (NT and NW) with the exception of TSC. TSC is normally related to a plant's ability to photosynthesize, therefore, some studies conducted on *V. unguiculata* seeds showed a reduction in TSC under NW, also in order to increase the number of free analytes able to gather water through osmosis [43]. Here, we found an increase in TSC in NW conditions. Maybe this could be related to the maintenance of a high photosynthesis rate, also without irrigation. As a matter of fact, NW is not always related to starch degradation in plant tissues [44]. These data further confirm a good resistance of this species against drought, a condition highly dangerous for many crop species not equally able to adapt to climate changes [45]. Finally, in terms of protein and amino acids, our analyses confirm that cowpea is an important source of these nutritional components and the growing condition did not affect their amount. Considering that in the Mediterranean area, especially in the Eastern and African sides, diets are deficient in terms of protein and amino acid

intake [16,46] and despite the change in food regimes that lead to a decrease of the intake of animal proteins, we estimate that cowpea could be a great support or even a crucial aliment to compensate these lacks. Moreover, the cowpea amino acidic content is two times higher than the widespread common bean *P. vulgaris* [16], therefore this species could be a good substitute for the traditional legume crops.

4.2. Conservative cultivation of V. unguiculata enhances soil fertility

As well documented, Fabaceae are able to accumulate organic nitrogen thanks to symbiotic interactions at the rhizosphere level. This phenomenon has positive effects also on soil fertility; however, soil management could also affect organic and inorganic components. For instance, CT is considered a major cause of soil C and N depletion as a consequence of soil organic matter mineralization [47]. NT has been widely indicated as a key strategy to increase C storage in arable soils. Our results are in line with this consideration as they show that converting CT to NT increased C stock by 2.58 Mg ha^{-1} on a silty clayey soil (Table 1), which means that NT increased the potential of soil to sequester C by 0.32 Mg ha^{-1} yr^{-1} under our experimental conditions. This is consistent with the previous findings of a review study by West and Post [48]. These authors reported that NT may increase C sequestration in soil by 0.20-0.57 Mg ha^{-1} yr^{-1}, according to the complexity of crop rotation. Also Aguilera et al. [49], in a recent meta-analysis reported a 0.44 Mg ha^{-1} yr^{-1} higher soil C sequestration under NT in the surface 34 cm of soil under a temperate climate.

Our findings also suggested that considering soil C expressed as concentration (g kg^{-1}) instead of as mass (Mg ha^{-1}) may lead to overestimating the role of NT for

soil C sequestration. However, we underline that we have data from the last eight years about the used NT soil patches, and our surveys showed an increase in soil C concentration in the 0–30 cm soil layer of, on average, 11% compared with CT. Conversely, soil C stock, which takes into account soil BD, was only 5% higher under NT than under CT. This is because lower BD of soil in the surface layers under NT (especially 0-5 cm) reduces the actual impact on soil C accumulation [18]. Nevertheless, NT did not reduce soil C stock in the subsurface soil layers (5–30 cm), and the net impact of NT on soil C stock in the 0–30 cm soil was positive.

Concerning nitrogen, the soil tillage could increase soil oxygen exposure and this promotes soil organic matter mineralization and soil N depletion [50]. Therefore, increasing soil organic matter in soils is a key way not only to increase soil C stock and mitigate climate change but also to enhance soil fertility and thus sustain food production [51].

As expected, our results showed that the evolution in soil N concentration and stock followed a similar pattern to that of soil C levels. Converting CT to NT led to a significant increase of soil N concentration in the 0–5 cm soil layer (Table 2). These results confirmed that variation of soil C and N levels as induced by NT differed considerably depending on the surface (0–5 cm) or subsurface (5–30 cm) soil layers [52]. Mazzoncini et al. [53] also found that soil C and N accumulated under NT may be mainly attributed to soil C and N variation in the topmost (0–10 cm) soil layer, which is in substantial agreement with our results (Tables 1 and 2). This is mainly due to the fact that NT limits the direct input of fresh organic matter to the subsoil, thus reducing the downward movement of soil organic matter, which is usually increased only in the surface 10 cm of soil. In addition, reducing soil disturbance decreases N mineralization and losses especially in the topmost soil layers [53], due

to a lower temperature [54] and aggregate turnover [55] in non-tilled soils than in tilled ones.

5. Conclusion

In conclusion, CA can offer many advantages in the Mediterranean context. First of these, is the saving of energy and costs. Studies performed on cowpea in a semiarid area of India showed that zero tillage practices provide a considerable energy saving (-17.1 GJ ha^{-1}) due to the lower input compared to CA [56]. The energy efficiency should be about 13 times higher in zero tillage systems than in conventional ones. However, Dixit et al. [56] showed that cowpea yield (intercropped with sorghum) was significantly higher in the context of conventional agriculture compared with NT.

Another key point of CA is the ability to reduce the impact on the greenhouse effect by anthropogenic gases emission. As suggested by Powlson [18] the total carbon sequestration in NT condition could reach about 0.3 Mg ha^{-1} yr^{-1}; our data confirm that the total CO_2 sequestration per year was estimated to be 0.32 Mg yr^{-1}.

This is due also to the usage of a cover crop mixture during the winter period which provided for better capture of the CO_2, while in Powlson [18] the meta-analysis took into account predominantly field not managed with cover crops. This underlines the importance of the integration of cover crops in zero tillage management.

Finally, concerning the ability of the soil to save water, our data suggested an increase of SOC in NT area against CT. Previous studies suggested that in the context

of the Mediterranean geographical area, an increase of about 0.4% SOC may lead to an increase of up to 34% of water saving [57].

Regarding all these elements and knowing that cowpea is a very interesting stress-tolerant minor crop with a short time maturity (about 60 days), cowpea could be introduced not only to relieve and reduce agricultural impact and climatic changes but also to supply a lot of vegetable-derived nutrients, like proteins and amino acids.

List of Abbreviations: CA: conservation agriculture; CT: conventional tillage; NT: no-tillage; W: watered; NW: not watered.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Evolution of daily precipitation (bars) and average daily temperature (line) of the field site during the course of the experiment.

Author Contributions: L.G., A.F., D.P., N.T. and A.G. designed and performed the field experiment, L.G., A.F., D.P., F.G., analysed data, E.T. and C.M. contributed during field activity, E.P., V.T., A.G. and M.L. supervised and contributed to the paper writing.

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Abbreviations: CA: conservation agriculture, CT: conventional tillage, NT: no-tillage; W: watered; NW: not watered.

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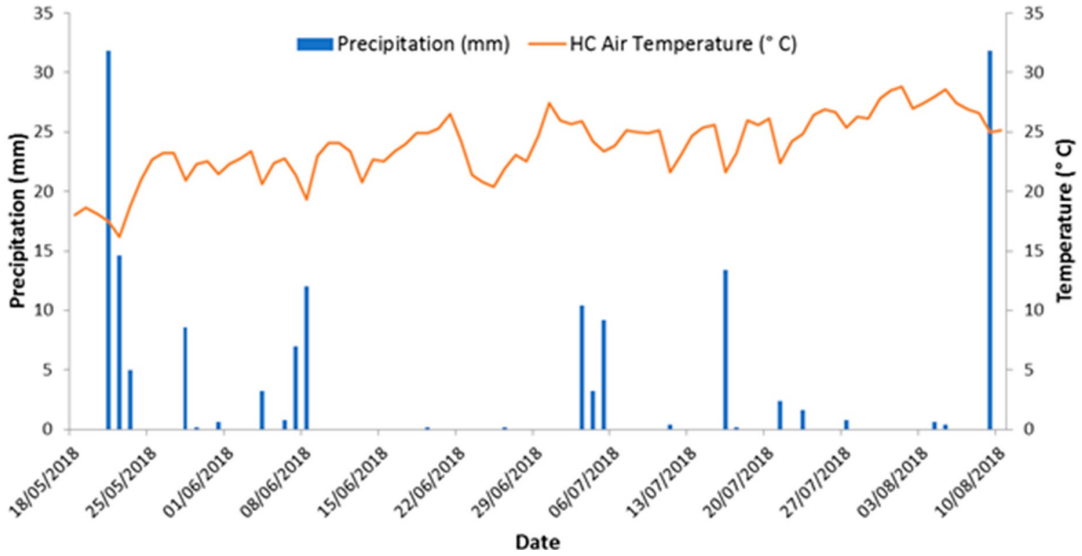

Figure S1: Evolution of daily precipitation (bars) and average daily temperature (line) of the field site during the course of the experiment.

3. Davide Panzeri & Lorenzo Guzzetti, Grazia Sacco, Gabriella Tedeschi, Simona Nonnis, Cristina Airoldi, Massimo Labra, Paola Fusi, Matilde Forcella, Maria Elena Regonesi (2020). Effectiveness of *Vigna unguiculata* seed extracts in preventing colorectal cancer. *Food Func, 11*(7), 5853-5865.

This work deals with the evaluation of the nutraceutical value of *V. unguiculata* seeds as a preventive agent to contrast the outbreak of colon malignancies by using *in vitro* cellular systems. The bioactivity of the phytocomplex is complemented with a detailed chemical characterization and the extract is progressively fractionated to identify the putative responsible for the cancer selective cytotoxic properties.

General considerations: In this work it was highlighted the occurrence of a small peptide acting as a trypsin and chymotrypsin inhibitor. This peptide is the most likely responsible for the selective cytotoxicity against colon cancer cell lines. Moreover, it is notable that the supplementation of *V. unguiculata* extract is able to reduce the amount of a common chemotherapy drug used for the treatment of colon cancer malignancies, allowing to hypotize that the consumption of *V. unguiculata* may be a possible dietary solution to reduce the amount of drug during a therapeutic cycle.

Effectiveness of *Vigna unguiculata* seed extracts in preventing colorectal cancer.

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Abstract

Colorectal cancer (CRC) is one of the most common types of cancer, especially in Western countries, and its incidence rate is increasing every year. In this study, for the first time *Vigna unguiculata* L. Walp. (cowpea) water boiled seed extracts were found to reduce the viability of different colorectal cancer (CRC) cell lines, such as E705, DiFi and SW480 and the proliferation of Caco-2 line too, without affecting CCD841 healthy cell line. Furthermore, the extracts showed the ability to reduce the level of Epidermal Growth Factor Receptor (EGFR) phosphorylation in E705, DiFi and SW480 cell lines and to lower the EC50 of a CRC common drug, cetuximab, on E705 and DiFi lines from 161.7 ng/mL to 0.06 ng/mL and from 49.5 ng/mL to 0.2 ng/mL respectively. The extract was characterized in its protein and metabolite profiles by tandem mass spectrometry and ^{1}H-NMR analyses. A Bowman-Birk protease inhibitor was identified within the protein fraction and was supposed to be the main active component. These findings confirm the importance of a legume-

based diet to prevent the outbreak of many CRC and to reduce the amount of drug administered during a therapeutic cycle.

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Introduction

Colorectal cancer (CRC) is one of the most frequent malignancy in the world. The outbreak of the majority of CRC forms is related to environmental factors, such as lifestyle and diet, while only a 5-10% of hereditariety is described[1]. During carcinogenesis and cancer progression, the up-regulation of survival signals is mainly responsible for the abnormal proliferation of CRC cells[2], in which the epidermal growth factor receptor (EGFR) signaling pathway is thought to play a crucial role. EGFR is a transmembrane tyrosine kinase receptor triggering two signaling pathways: the RAS-RAF-MAPK axis involved in cell proliferation and the PI3K-PTEN-AKT pathway involved in cell survival and escaping from apoptosis[3]. In

the last decades, many drugs have been developed for the treatment of CRC. Among these, the monoclonal antibodies (MoAbs) cetuximab and panitumumab bind to the extracellular domain of EGFR when it is in the inactive configuration, compete for receptor binding by occluding the ligand-binding region, and thereby block ligand-induced EGFR activation, inducing its internalization and degradation[4]. However, these drugs are expensive and still characterized by some side effects such as severe skin toxicity, occurring in approximately 80% of patients[5], corneal erosion[6], headache, pulmonary damages, general weakness and diarrhea[7]. Moreover, they show efficacy in no more than 30% of patients[8]. Indeed, it has been demonstrated that hyperactivating mutations occurring in downstream effectors (such as KRAS, NRAS and BRAF) represent the main mechanism of primary resistance. However, even in patients without any downstream mutations, the percentage of efficacy of anti-EGFR MoAbs is less than 50%[9-18]. All these concerns underline the need to identify new approaches, such as the application of nutraceuticals able both to elicit and expand the range of chemo-preventive actions, while reducing the amount of administered drugs during a therapeutic cycle[19]. Fruit, vegetables and other edible plant parts are the primary sources for human nutrition and medicine[20]. The increasing knowledge on plant biodiversity and biotechnology has dramatically changed the role of plant food on human health

and wellbeing. Nutritional therapy and phytotherapy have emerged as new concepts and healing systems have quickly and widely spread in recent years. Considering that plant foods easily reach the stomach and the gut, these organs represent the most suitable targets to estimate the biological activities of food phytocomplexes[19].

Pulses have received increasing attention in the last decades due to their nutraceutical properties, such as antioxidant, anti-inflammatory, hypoglycemic and other activities[21,22]. Moreover, several studies have shown pulses anticancer properties related to the presence of specific classes of phytochemicals, such as resistant starch fermenting in the gut and being converted into SCFAs, proteins, like amylase and protease inhibitors, globulins and polyphenols[23].

In Europe, the production of pulses is lower than in other continents and mainly limited to peas, chickpeas and faba beans, while in poor and developing countries other species and local cultivars are preferred due to higher accessibility[24]. Among these minor crops, *Vigna unguiculata* L. Walp., also known as cowpea, stands out due to its adaptability to harsh environmental conditions, such as drought and minimum field tillage[25].

Its seeds provide high amount of proteins, peptides, amino acids and other minor metabolites such as folates and minerals (calcium, zinc and iron)[23]; leaves are also

sometimes consumed fresh or boiled increasing the uptake of polyphenols and fibers in diet[26,27]. Although the nutritional traits of this species is well documented, relatively little is known about the anticancer properties of this species, mainly due to bioactive peptides and polyphenols but results are controversial and require further investigations[28,29]. Therefore, in this study *V. unguiculata* beans phytoextract was considered to evaluate potential anticancer activities. Specifically, phytochemical analyses were combined with bioactivity investigations to clarify the role of specific bean components as a possible supplement in EGFR-targeted therapies for CRC.

Experimental

Plant material and phytoextraction

V. unguiculata seeds from three batches were collected (Colfiorito, Italy, batches 17117 and 18039, and Castellani, Italy, batch 011018). Seeds were water boiled for one hour and left resting one more hour in water at room temperature (RT). This treatment aimed at emulating the typical boiling process of conventional food recipes.

Seeds were then incubated overnight at 50°C to dry completely and then they were grinded into a powder. Two grams of dry powder were extracted in 50 mL of milliQ

water at RT with a magnetic stirrer for 5 minutes and then centrifuged at 5000g for 30 minutes at RT. Supernatants were then recovered and freeze-dried.

Separation of phytoextract components

Molecular weight-based separation

Ultra-2 mL Amicon filters (Merck-Millipore, Germany) with a cut-off of 3 kDa were used to begin separation process of extracts. Freeze-dried extracts were resuspended in water at a concentration of 40 mg/mL. After conditioning tubes with 2 mL of water, extracts were loaded and centrifuged till complete separation at 7197g at 4°C. The upper and the lower fractions were collected, freeze-dried and stored at -20°C. Protein content was determined using Coomassie brilliant blue G-250 method (Thermo Scientific Rockford, IL, USA) and using bovine serum albumin (BSA) as standard protein for calibration curve.

DEAE chromatography

DEAE chromatography was performed to further purify bioactive components. The upper fraction was resuspended in 5 mM Tris HCl pH 8 to reach a concentration of 40 mg/mL . 1 mL of DEAE resin (DEAE Sephacel, GE-Healthcare, USA) was centrifuged at 5000g for 10 minutes at 4°C to separate the resin from ethanol. Ethanol was removed, then 10 mL of water were added, centrifuged and

supernatant was removed. The resin was further resuspended in 10 mL of 5 mM Tris HCl pH 8 for conditioning and centrifuged, then about 400 mg of sample were loaded and incubated on rotating wheel for 60 minutes at 4°C.

The mixture was subsequently loaded onto a column at a flow rate of 1 mL/min and eluted with 10 mL of 5 mM Tris HCl pH 8, 0.5 M NaCl at the same flow rate. The flowthrough and the eluted fractions were recovered and stored. Eluted fractions were dialyzed against PBS buffer (25 mM phosphate buffer pH 7.2, 0.15 M NaCl) with a 13000 Da cut-off membrane overnight at 4°C.

Size exclusion chromatography

A further purification was carried out using the AKTA Purifier Instrument (GE-Healthcare) equipped with a Superose 12 10/300 GL gel filtration column (GE Healthcare, Life Sciences, Little Chalfont, England), pre-equilibrated with PBS buffer. Elution was performed at a flow rate of 0.5 mL/min in the same buffer. A calibration curve was obtained by plotting elution volume parameters of a set of standard proteins against the logarithm of their molecular weights. Standards employed at 1 mg/mL: Immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), bovine β-lactoglobulin (35 kDa) and bovine cytochrome C (12.7 kDa) (Sigma Aldrich, St. Louis, MO, USA).

Removal of the hydrophobic components

A further step of purification was carried out on the fractions isolated through the size exclusion chromatography by using SPE C-18 Bond Elute cartridges (Agilent Technologies, USA) in order to remove the hydrophobic components. Samples were passed through the cartridge and the unbound fraction was recovered.

Proteomic analysis

HPLC fractions were reduced, derivatized and digested with trypsin (protein: protease ratio 20:1) as described in[30] before MS/MS analysis.

Peptides separation was achieved on a Thermo Easy-nLC 1000, and MS data were acquired on a Thermo Q-Exactive–HF, with a data-dependent top 15 method, the survey full scan MS spectra (300–1650 m/z) were acquired in the Orbitrap with 60000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra resolution was set to 15000, AGC target 1e5, IT 80 ms; normalized collision energy 28 and isolation width of 1.2 m/z.

Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with Sequest algorithm against the proteome of NCBI Phaseoleae (release 05th August 2019) with minimum peptide length 6 amino acids, carbamidomethylation as fixed modification, Met oxidation and Arg/Gln deamidation as variable modifications[31].

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017846.

^{1}H-NMR metabolic profile

The total cowpea water boiled seed extract was suspended in H_2O:D_2O (9:1) at a final concentration of 10 mg/mL. 3-(Trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt (TSP, final concentration 0.5 mM) was used as external reference and alanine doublet at 1.48 ppm as internal reference for chemical shift. The pH of the sample was verified with a microelectrode (Mettler Toledo, Columbus, OH, USA) and adjusted to 7.4 with NaOD and DCl. The acquisition temperature was 25 °C. All spectra were acquired on an Avance III 600 MHz NMR spectrometer (Bruker, Billerica, MA, USA) equipped with a QCI (^{1}H, ^{13}C, ^{15}N/^{31}P and ^{2}H) cryogenic probe. ^{1}H NMR spectra were recorded with *noesygppr1d* pulse sequences (Bruker library) and 256 scans, spectral width 20 ppm, relaxation delay 5 s. They were processed with 0.3 Hz line broadening, automatically phased and baseline corrected. The ^{1}H,^{1}H-TOCSY (Total Correlation SpectroscopY) spectra were acquired with 24 scans and 512 increments, a mixing time of 80 ms and relaxation delay of 2 s. ^{1}H,^{13}C-HSQC (Heteronuclear Single Quantum Coherence) spectra were acquired with 48 scans and 256 increments, relaxation delay 2 s. The NMR data were processed using

MestreNova 14.1.0 software (Mestrelab Research, Santiago de Compostela, Spain). Compound identification and assignments were done with the support of 2D NMR experiments and comparison with reported assignments.

Bioactivity assessment

Cell cultures

CCD841 (ATCC® CRL-1790™) human healthy mucosa cell line and CaCo-2 (ATCC® HTB-37™) human colorectal cancer cell line were grown in EMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin. E705 (kindly provided by Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy) and SW480 (ATCC® CCL-228™) human colorectal cancer cell lines were grown in RPMI 1640 medium supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. DiFi human colorectal cancer cell line, kindly provided by Dr. Josep Tabemero (Vall d'Hebron Institute of Oncology, Barcelona, Spain), was grown in Ham's F12 medium supplemented with heat-inactivated 5% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. ATCC cell lines were validated by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem

repeat loci and amelogenin (for gender identification). All the reagents for cell cultures were supplied by Lonza (Lonza Group, Basel, Switzerland).

Viability assay

Cell viability was investigated using MTT-based *in vitro* toxicology assay kit (Sigma, St. Louis, MO, USA), according to manufacturer's protocols.

In detail, the different cell lines were seeded in 96-well microtiter plates at a density of 1×10^4 cells/well, cultured in complete medium and treated after 24 hours with increasing concentrations of total extract (0-4000 µg/mL). In order to evaluate the combined effect of Cowpea extract and cetuximab, 24 hours after the seeding the cells were treated with different concentrations of cetuximab (0-100 µg/mL) and at fixed concentrations of cowpea total extract (200 and 1000 µg/mL).

After 48 hours at 37°C, the medium was replaced with a complete medium without phenol red containing 10 µL of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). After 4 hours incubation more for CCD841 and 2 hours for CRC cells lines, formazan crystals were solubilized with 10 % Triton X-100, 0.1 N HCl in isopropanol and absorbance was measured at 570 nm using a microplate reader. Cell viabilities were expressed as a percentage against untreated cell lines used as controls. Before each experiment the extract or the related fractions were filtered through a nitrocellulose 0.22 µm filter and the protein

concentration was evaluated by using Coomassie brilliant blue G-250 (Thermo Scientific Rockford, IL, USA) and BSA as a standard protein.

Proliferation assay

In order to evaluate the effect of cowpea total extract on the cellular proliferation of the CRC cell lines, the cells were counted at consecutive time points. The cells were seeded in 35 mm dish at a density of 1-2×10^5, treated with the extract (200 and 2000 μg/mL) 24 hours after seeding and harvested by trypsinization at 24, 48 and 72 hours after treatment. Aliquots of the cell suspension were counted in Burker's chamber. All counts were expressed as total number of cells.

SDS–PAGE and Western blot

To examine the effect of extract on the EGFR phosphorylation, the CRC cell lines and the healthy cell line were seeded at 75×10^4 cells/60 mm dish and treated for 48 hours with the total extract at 200 and 2000 μg/mL. The cells were rinsed with ice-cold PBS and lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS), containing protease and phosphatase inhibitors and 1 mM PMSF. Homogenates were obtained by passing 5 times through a blunt 20-gauge needle fitted to a syringe and then centrifuged at 15000 xg for 30 minutes. Supernatants were analyzed for protein content by the BCA protein assay[32]. SDS-PAGE and Western blot were carried out by standard procedures[33].

Twenty or sixty micrograms of proteins were separated on 10% acrylamide/bis-acrylamide SDS-PAGE, transferred into a nitrocellulose membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% (w/v) dried milk in PBS for 30 minutes at RT and then probed overnight at 4°C with the appropriate antibodies in 5% (w/v) BSA in PBS. After three 10 minutes washes with PBS containing 0.1% (v/v) Tween 20 (PBS-T), membranes were treated for 1 hour at RT with an HRP-conjugated secondary antibody diluted in 5% (w/v) dried milk in PBS. After three washes in PBS-T, detection was performed using an ECL plus detection system (Millipore, Billerica, MA, USA). Protein levels were quantified by densitometry of immunoblots using Scion Image software (Scion Corp., Frederick, MD, USA). The following primary antibodies (all purchased from Cell Signaling Technology, Danvers, MA, USA) were used: anti EGFR (dilution 1:1000), phospho-EGFR (Tyr1068; dilution 1:1000), p44/42 MAPK (ERK 1/2; dilution 1:1000), phospho-p44/42 MAPK (ERK 1/2) (Thr202/Tyr204; dilution 1:1000), Akt (dilution 1:1000), phospho-Akt (Ser473; dilution 1:1000), GAPDH (dilution 1:10000) and vinculin (dilution 1:10000). IgG HRP-conjugated secondary antibodies (purchased by Cell Signaling Technology, Danvers, MA, USA) were diluted 1:10000.

Statistical analyses

Statistical analyses were performed by using software R, version 3.3.3. Packages used for the analyses and the graphs were lme, lme4, nlme, glmmTMB and ggplot2. The threshold of statistical significance was set at 0.05.

MTT assay

To analyse the impact of the amount of extracts on the viability of the different cell lines a Generalized Linear Mixed Effects Model (GLMM) was used. The response variable (% cells survival) was assumed to be binomial or beta-binomial distributed in case of overdispersion[34]. Fixed effect analysed was the concentration of extract in interaction with the cell line. Since three different batches were tested, they were treated as a random effect.

Proliferation assay

Data from proliferation assays were analysed by a GLMM. Response variable was the number of cells and it was assumed to be negative-binomially distributed. Fixed effect was growth time up to 72 hours after the treatment in interaction with the amount of extracts used for the treatment itself. For each cell lines, 3 replicates were performed and therefore treated as random effect.

Combination experiment between cetuximab and total extract

One Way ANOVA was used to evaluate the effect of the supplementation of two different concentrations of extract on the EC50 of cetuximab. In order to compare

the effect of the supplementation of extract at different concentrations (200 µg/mL and 1000 µg/mL) against the control (cells treated only with cetuximab) a Dunnett test was performed.

Densitometric analysis

A Linear Model (LM) was performed to evaluate statistical differences regarding EGFR phosphorylation among the different extract concentrations in the 5 cell lines. The response variable was the level of EGFR phosphorylation while the categorical variable was the interaction between the cell line and the concentration of extract. The same analysis was performed on ERK and Akt phosphorylation levels.

Evaluation of protein-dependence

To verify the existence of a relationship between CCD841 and E705 cell viability and the amount of proteins in the samples, a GLMM was carried out considering a binomial distribution of the response variable and proteins amount in interaction with the cell line as fixed effect. Both total and purified extracts were taken into account, so that the different extract fractions (see paragraph 2.2) were considered the random component within the model.

Results & discussion

Effect of *V. unguiculata* extract on the viability of healthy and colorectal cancer cell lines

Starting from 2 g of dried seeds from 3 different batches the following extract yields were obtained: 102.4 ± 11 mg from the first batch, 203 ± 9 mg from the second and 81.8 ± 3.78 mg from the third. The protein content was equal to 4.168 ± 0.379 mg for the extraction of the first batch, 3.179 ± 0.203 mg for the second batch and 8.142 ± 0.407 mg for the third. Extracts of *V. unguiculata* from the three different batches were tested on healthy mucosa and CRC cell lines with different molecular profiles: Caco-2, E705 and DiFi cell lines, all wild type for EGFR, KRAS, NRAS, and BRAF genes, as well as SW480 cell line, carrying the KRAS G12V mutation and wild-type for the other aforementioned genes. The DiFi cell line is characterized by a strong EGFR gene amplification profile. Fig. 1 reports the results of MTT assay on each cell line at different phytoextract concentrations. Data suggest a dose-dependent effect in E705 ($p < 0.001$), DiFi ($p < 0.001$) and SW480 ($p < 0.001$) cell lines, with a percentage of viability at 2000 μg/mL of 50%, 23% and 45%, respectively. Neither Caco-2 cancer cell line ($p = 0.672$) nor the healthy one CCD841 ($p = 0.301$) were affected by the treatment.

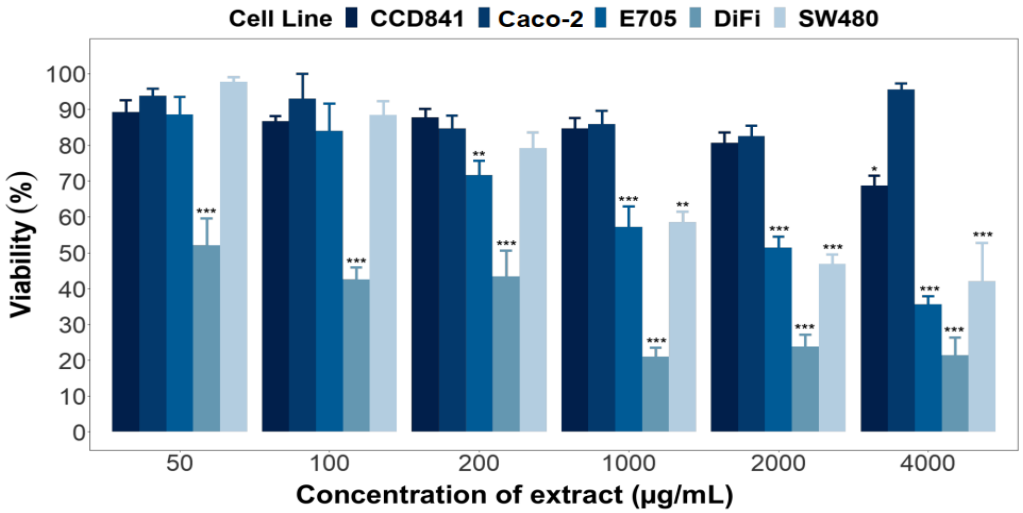

Fig. 1 Effects of *V. unguiculata* extract after 48 hours treatment on the viability of colon cell lines. Data are expressed as the mean percentage of viability of the three batches tested compared to the untreated control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, with Bonferroni's correction). The experiment was performed with increasing concentrations of phytoextract (from 50 µg/mL to 4000 µg/mL).

Proliferation assays on healthy and CRC cell lines after extract treatment

To evaluate the cytostatic effect of the phytoextract, proliferation assays were performed on healthy and CRC cell lines treated with 200 and 2000 µg/mL extract at different times. Results showed a general cell growth decrease of the CRC lines (Fig. 2). In particular, the effect on Caco-2 was detected only at the highest dose after 60 hours treatment, whereas E705 proliferation showed a significant effect

already after 30 hours at both concentrations. Noteworthy, at a concentration of 2000 µg/mL, DiFi cells did not show any growth. Concerning SW480, the reduction in the growth rate at 2000 µg/mL extract was highly significant compared to the untreated control approximately after 60 hours treatment (confidence bands do not overlap). The healthy line CCD841 was found not to be affected by the treatment with the extract at any concentration tested (Fig. 1, Supplementary information). Therefore, our data indicate that components of *V. unguiculata* seed extract may play a role in cancer prevention, especially by slowing down cancer cells proliferation.

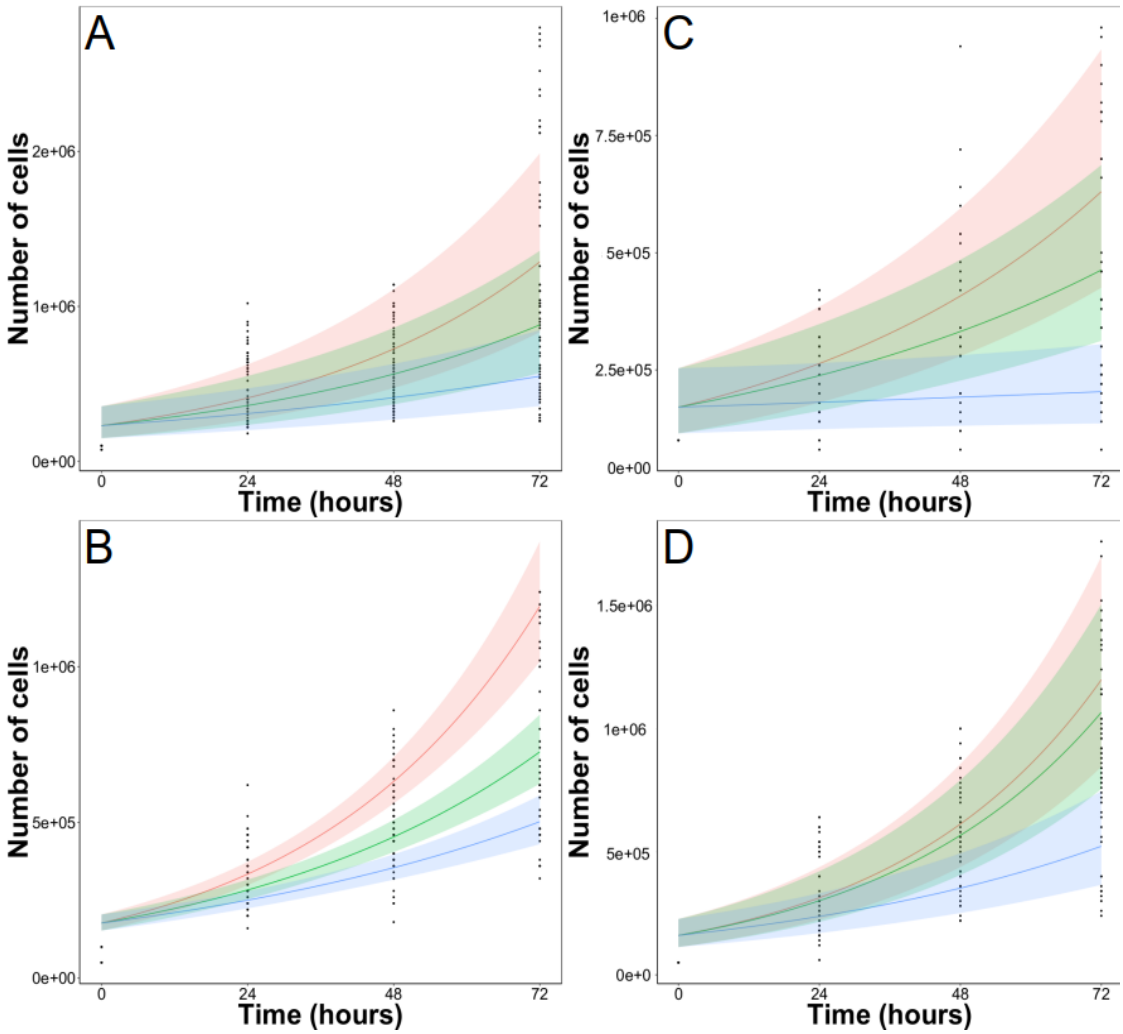

Fig. 2 Cell proliferation. CRC cell lines treated after 24 hours after seeding, harvested and counted at 24, 48 and 72 hours after treatment. A: Caco-2, B: E705; C: DiFi; D: SW480. Coloured lines represent the growth function in time of cells treated with different concentration of extract (red: control, green: 200 μg/mL,

blue: 2000 µg/mL). Bands show the 95% confidence intervals. When bands overlap, there is no significant difference among treatments.

Analysis of EGFR phosphorylation and related downstream pathways in response to phytoextract

As EGFR signaling is one of the pathways mainly involved in CRC pathogenesis, the activation of EGFR and of the main downstream effectors (ERK for the MAP kinase pathway, Akt for the PI3k-Akt-mTOR axis) were evaluated through western blot and densitometric analysis after treatment with 200 µg/mL and 2000 µg/mL extract (Fig. 3). Results showed that in E705 cells the level of phospho-EGFR significantly decreased at 200 µg/mL and even more at 2000 µg/mL extract ($p < 0.01$). DiFi and SW480 showed a decrease in phospho-EGFR levels only at 2000 µg/mL ($p < 0.01$). Conversely, no effects were shown on Caco-2, neither at 200 µg/mL nor at 2000 µg/mL. As expected, no EGFR phosphorylation was found in CCD841. All CRC cell lines displayed high phospho-EGFR levels compared to healthy control[35].

The precise interplay of the two EGFR downstream pathways is still unknown, but most data suggest that the MAP kinase pathway is the most relevant one[8,36,37]. As a matter of fact, the presence of mutations able to hyperactivate this pathway (such

as those occurring in KRAS, NRAS and BRAF genes) is the main factor for ruling out EGFR-targeted therapies, that have been developed in the last 10 years[9].

However, concerning the two EGFR downstream pathways, our data reveal that the situation is more complex. Indeed, ERK activation was downregulated only in the DiFi cell line in a dose-dependent manner ($p < 0.001$), while no variations in Caco-2, E705 and SW480 cells were detected. Diversely, a significant decrease in Akt activation was observed in DiFi cell line already at 200 µg/mL extract, while in E705 and SW480 cell lines only at the highest extract dose. In general, the responses of these cell lines were found to be concentration-dependent (E705 $p=0.028$, DiFi $p < 0.001$, SW480 $p=0.009$). A decrease in ERK ($p < 0.001$) and Akt ($p = 0.001$) activation was also observed in the CCD841 normal mucosa cell line, but only at the highest phytoextract concentration and with limited evidences at the viability and proliferation level (Fig. 1 and Fig. 1, Supplementary Information). Overall, the alterations of the activation levels of EGFR and partly of its downstream effectors are in line with the viability and proliferation assays. In E705 and DiFi cell lines, both sensitive to anti-EGFR therapies, the decrease of EGFR phosphorylation and the decrease of ERK and/or Akt activation led us to hypothesize a putative supplementary effect between *Vigna unguiculata* extracts and EGFR-targeted therapies.

The SW480 cell line is characterized by the KRAS G12V mutation and presents a constitutively activated MAP-kinase pathway, so that the significant decrease in EGFR phosphorylation can lead to the reduction of the PI3K-mTOR pathway with a decrease of Akt phosphorylation, leading cells to apoptosis. We cannot exclude that cell proliferation may be mediated by alternative, MAP-kinase independent pathways. The Caco-2 cell line shows a decrease in cell proliferation only at the highest dose but no alterations in the activation of EGFR and its downstream effectors are observed: for this cell line, that is characterized by a complete RAS-BRAF wild-type status, we can hypothesize that the driver is outside the EGFR pathway but acts on it through a putative dimerization of EGFR with other members of the EGFR family[38]. Therefore the phytoextract plays a minor role, in accordance with the smaller decrease observed in cell proliferation. As for the normal mucosa cell line, the decrease of downstream pathways activation at the highest dose does not lead to any alteration in the viability and proliferation levels. It is conceivable that the administration of *Vigna unguiculata* extract treatment may have no effect on normal mucosa cells. This is an extremely important factor to avoid the tedious side effects of the majority of chemotherapies.

Overall, our Western blotting results clearly indicate that not only the MAP kinase axis, but both EGFR downstream pathways are relevant. At clinical level, as

mentioned before, the presence of mutations able to hyperactivate this pathway, such as those occurring in KRAS, NRAS and BRAF genes, is the main factor for ruling out EGFR-targeted therapies[9]. On the contrary, little is known about the P3K-Akt-mTOR pathway, if we exclude few, sporadic studies that investigated PIK3CA mutations and PTEN protein deregulations, but without an extensive confirmation by the wide scientific community[39,40,41]. Having in mind that only up to 30% of RAS-BRAF wild-type cases may profit from the administration of EGFR-targeted therapies, our data suggest a deeper investigation of the P3K-Akt-mTOR axis.

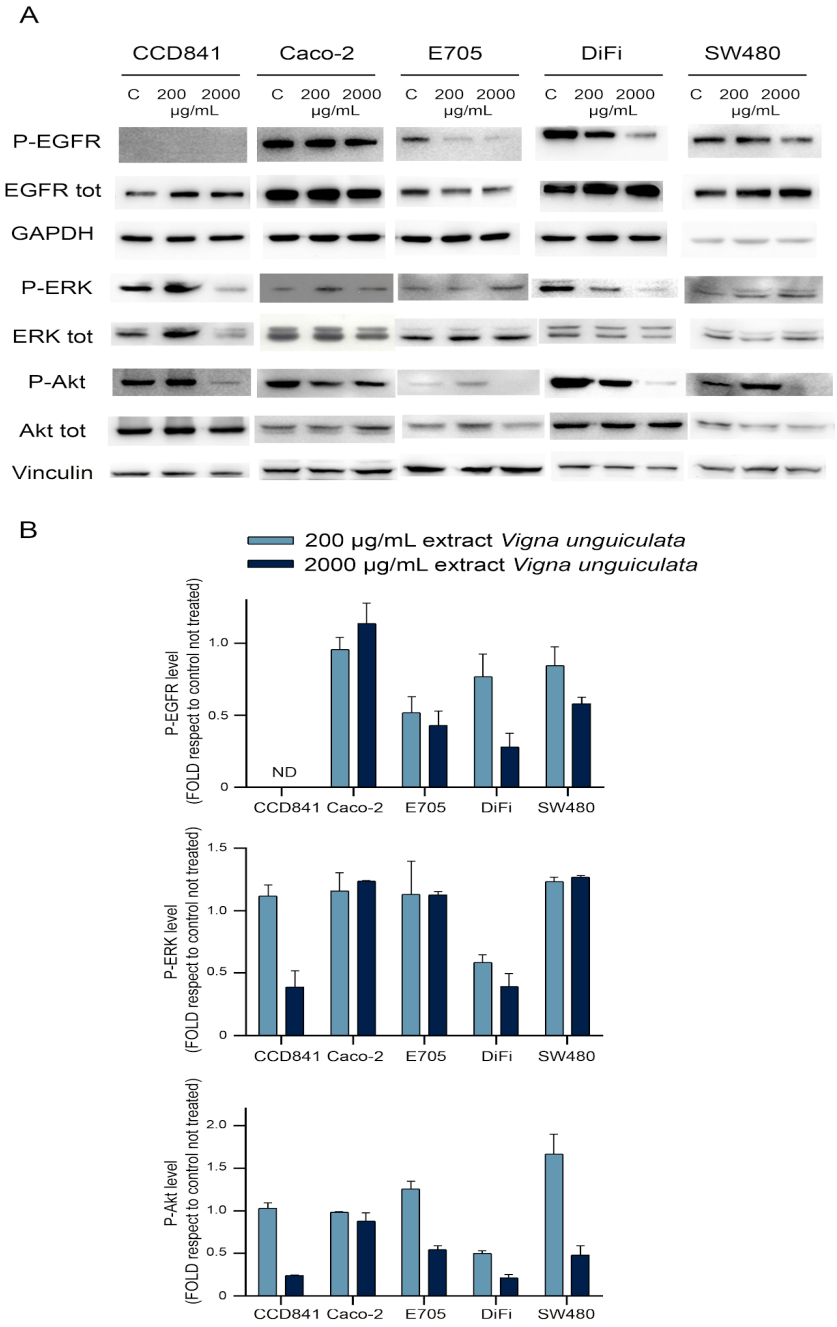
A
CCD841
Caco-2
E705
DiFi
SW480
C 200 2000 µg/mL
P-EGFR
EGFR tot
GAPDH
P-ERK
ERK tot
P-Akt
Akt tot
Vinculin
B
200 µg/mL extract Vigna unguiculata
2000 µg/mL extract Vigna unguiculata
P-EGFR level (FOLD respect to control not treated)
ND
P-ERK level (FOLD respect to control not treated)
P-Akt level (FOLD respect to control not treated)
CCD841 Caco-2 E705 DiFi SW480

Fig. 3 Western blotting analysis of EGFR, ERK and Akt phosphorylation in CRC and normal mucosa cell lines. (A) Representative Western blotting performed on protein extracts (20 μg for DiFi cell line and 60 μg for the other cell lines in EGFR analysis and 60 μg for all cell lines in ERK and Akt analysis), using anti-P-EGFR, anti-EGFR, anti-P-ERK, anti-ERK, anti-P-Akt and anti-Akt antibodies. GAPDH and vinculin was used as loading control. (B) Determination of phosphorylation rate by densitometric analysis was performed with Scion Image Software. Data are expressed as the phospho/total ratio and each ratio is normalized on phospho/total ratio of not treated cell line.

Extract ability to supplement cetuximab treatment

The significant reduction in EGFR activation may propose these extracts as potential co-drugs to be administered in combination with EGFR-targeted therapies, that have some adverse side effects and are effective in only up to 30% of treated patients. The ability of the extract to complement the cetuximab EGFR-targeted therapy was evaluated. CRC cell lines were treated with cetuximab (0-100 μg/mL) and 200 μg/mL and 1000 μg/mL extract (Fig. 2, Supplementary information). These concentrations were selected on the basis of extract effect on the two drug-sensitive cell lines, i.e., E705 and DiFi. As far as E705 line is concerned, results show

that the EC50 of the drug decreased from 161.7 ± 18.3 ng/mL to 18.4 ± 9.8 ng/mL when E705 were treated with 200 μg/mL and to 0.06 ± 0.01 ng/mL in combination with 1000 μg/mL of extract. Regarding DiFi cell line, a similar pattern to that of E705 was observed. Specifically, the EC50 of the drug decreased from 49.5 ± 1.0 ng/mL to 12.4 ± 5.4 ng/mL in combination with 200 μg/mL of extract and to 0.2 ± 0.1 ng/mL with a treatment of 1000 μg/mL of extract. In both cell lines, differences between treatments and control were statistically relevant (p <0.05). We assume the possible use of lower doses of drug in combination with cowpea extracts in the treatment of patients. A diet regime including pulses' consumption, such as cowpea, could be a turning point in targeted therapies: there are, in facts, evidences showing that consumption of pulses is linked to a lower CRC incidence rate[42-46]. Furthermore, some studies showed that it would be enough to include in the diet the consumption of approximately 100 g of legumes per week to prevent many forms of cancer, including CRC[47].

Chemical characterization of the total extract (^{1}H-NMR)

The metabolic profiling of the total extract was characterized by NMR spectroscopy data exploited for primary and secondary metabolite identification following the approach developed for the analysis of complex plant extracts[48-53]. The

identification of metabolites was based on the analysis of mono and bidimensional NMR spectra and is in agreement with data from previous literature[54]. Overall, ^{1}H-NMR profile (Fig. 4) revealed the presence of amino acids (alanine, valine, serine, threonine, methionine, cysteine, arginine, aspartate, glutamate, proline, glycine, tryptophan, tyrosine, phenylalanine), organic acids (acetate, lactate, citrate, succinate, GABA, nicotinic acid), sugars (glucose, sucrose, raffinose among the most abundant), choline and uracil. In addition to previously reported results[54], we also observed trigonelline and a significant amount of broad resonances (indicated by red arrows in Fig. 4), corresponding to proteins, as confirmed also by typical TOCSY correlations in the amide NH region (Fig. 3, Supplementary information).

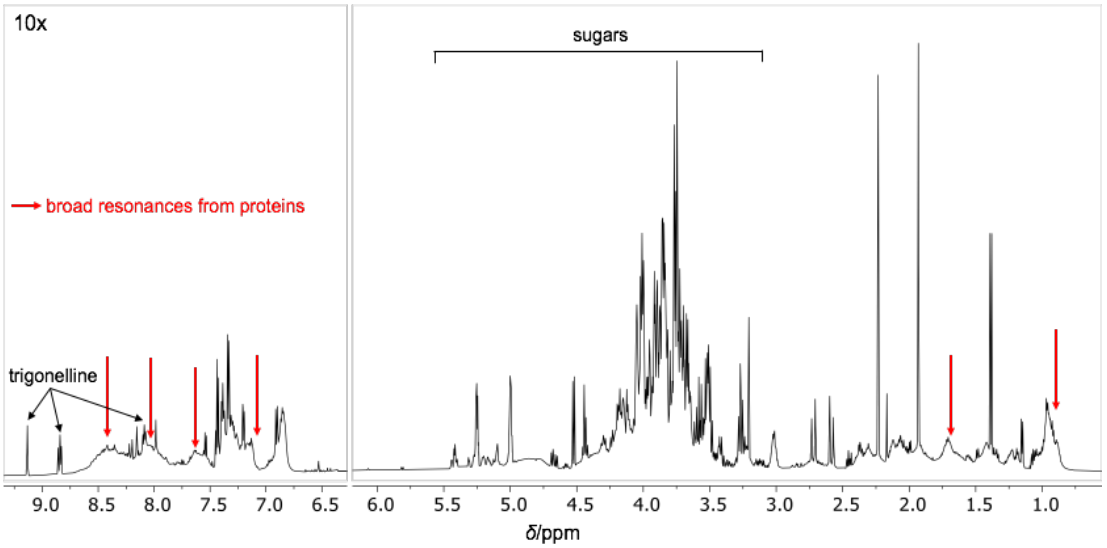

Fig. 4 ^{1}H-NMR profile of a total cowpea water boiled seed extract sample dissolved in H_2O:D_2O 9:1 at a final concentration of 10 mg/mL, 25 °C, 600 MHz.

Fractionations of the bioactive components

To identify the molecule/s responsible for the selective cytotoxicity against CRC cells, the extract was progressively fractionated and tested to follow the bioactive component. The effects of the different fractions are expressed as the viability ratio between E705 cancer cells and CCD841 healthy cell line (Fig. 5A). Only fractions showing a viability ratio below 1 were considered active and further purified. At first, the separation through Centricon suggested that the bioactive component is a compound with a molecular weight higher than 3 kDa. DEAE chromatography and size exclusion chromatography (Fig. 4, Supplementary information) showed that it

is a macromolecule with an overall negative charge at physiological pH, with an apparent molecular weight ranging from 8 kDa to 20 kDa. The P1, P2 and P3 fractions obtained by SEC were further loaded onto SPE C-18 Bond Elute cartridge to remove hydrophobic compounds. The active fraction (P1 plus P2) was effective also on the other sensitive CRC lines (Fig. 5B).

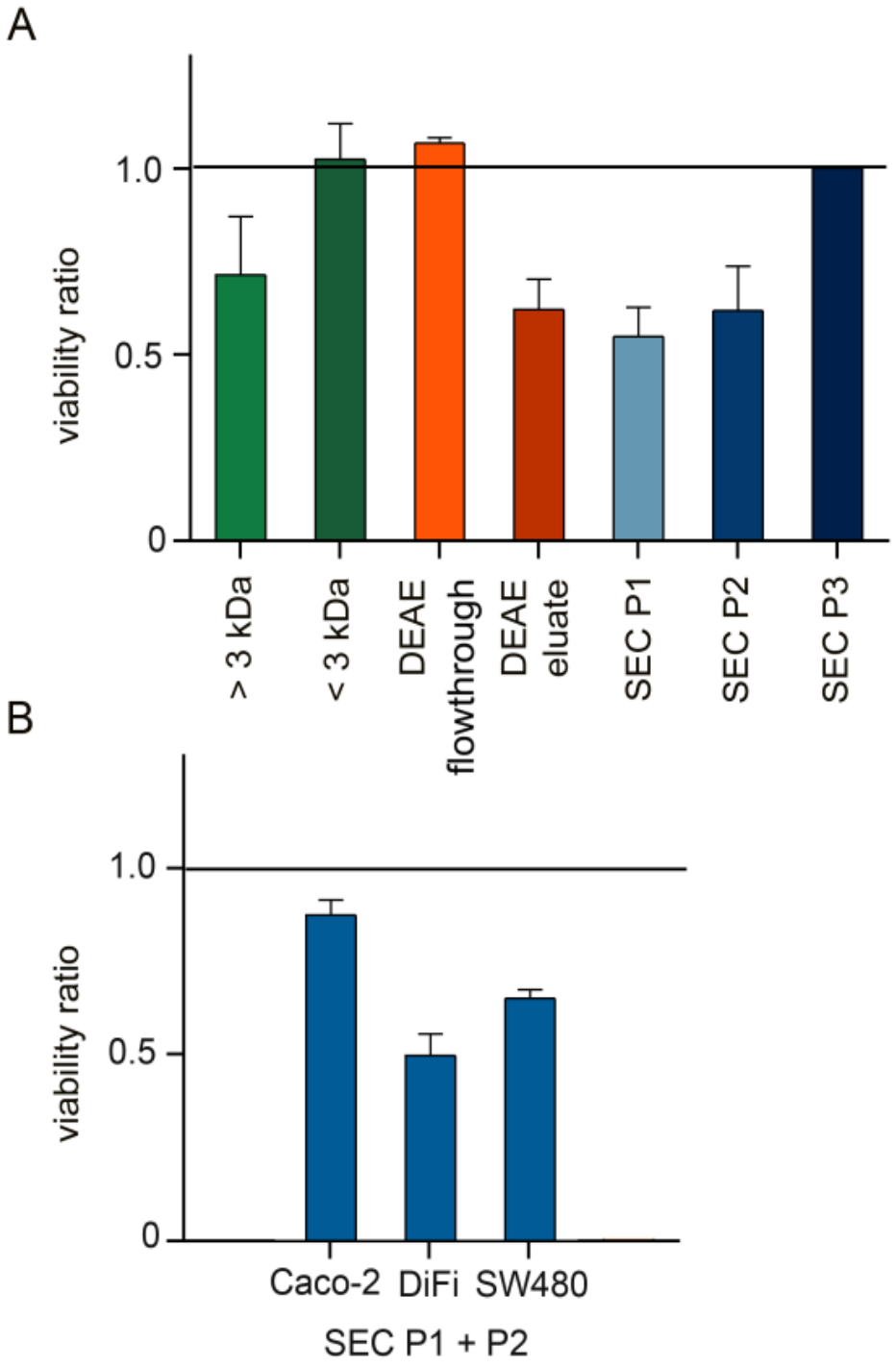
A
viability ratio
1.0
0.5
0
> 3 kDa
< 3 kDa
DEAE flowthrough
DEAE eluate
SEC P1
SEC P2
SEC P3
B
viability ratio
1.0
0.5
0
Caco-2
DiFi
SW480
SEC P1 + P2

Fig. 5 Viability ratio of E705 compared to CCD841 cells treated with the different fractions obtained by the progressive steps of purification (3 kDa cut-off filter, DEAE chromatography and P1, P2, P3 of the SEC) (A). Viability ratio of Caco-2, DiFi, SW480 compared to CCD841 cells treated with the SEC-purified fraction (P1 plus P2) (B). Values are mean ± SEM.

Protein-dependent selective cytotoxic properties of the extract

The abovementioned purification procedures led to obtain a set of samples with a variety of protein concentrations within the phytocomplex, so that E705 and CCD841 cells were treated with a wide range of samples with different protein concentrations (from 0 to 115 μg/mL). To test the effect of protein concentration on the viability of E705 and CCD841 cells a regression model was set up (see Experimental).

Results highlighted a clear negative effect of protein concentration on the viability of E705 cells with significant effects already at a concentration around 30 μg/mL, while in the healthy cell line the effect was much lower and detectable only at doses higher than 90 μg/mL (Fig. 6). Therefore, proteins appeared to be the effective component in the selective cytotoxic activity of the extract.

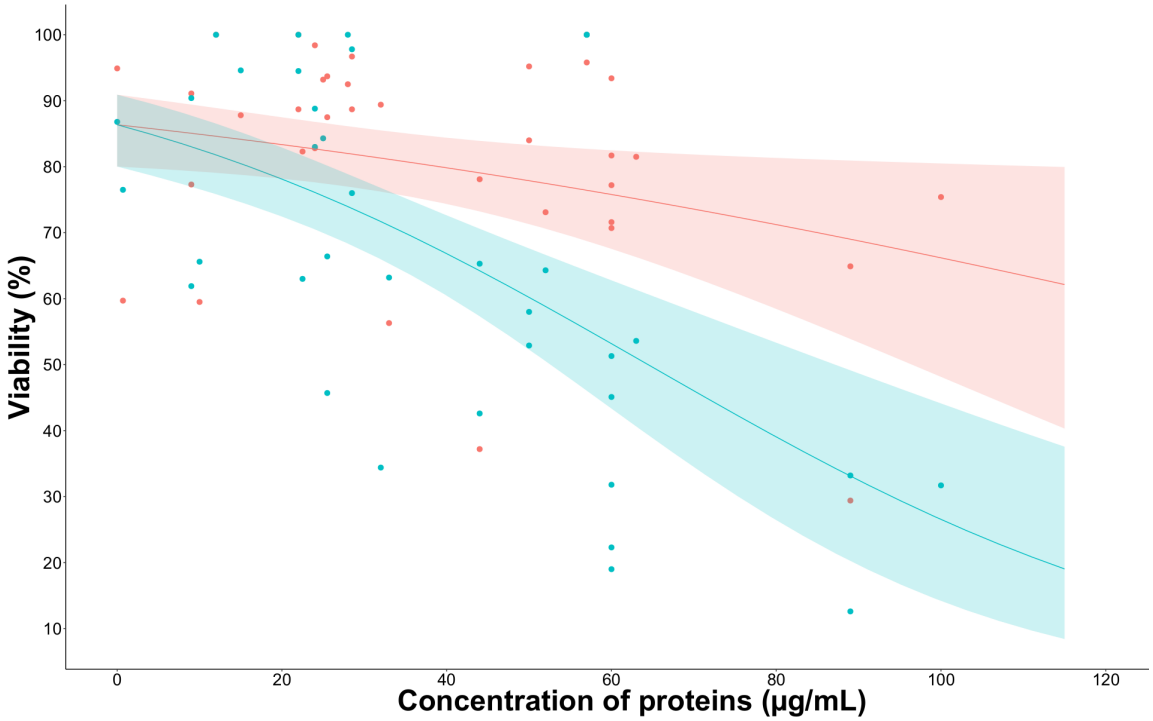

Fig. 6 Model showing the effect of protein amount on CCD841 (red line) and E705 (blue line) cell lines.

Proteomic assessment of bioactive fractions

The protein composition of all the fractions separated by SEC was characterized by LC-MS/MS using a shotgun proteomic approach, a gel free technology that allows to identify all the proteins in a sample by obtaining sequence information without

previous purification of each single protein[55]. The differential analysis between the active fraction data (P1 plus P2) and fraction P3, which did not show any biological activity, allowed to identify the proteins exclusively present in P1+P2 fractions, most probably responsible for the bioactivity. The analysis was repeated on two different preparations to verify the reproducibility of the results. The list of these proteins is reported in Table 1.

Accession	Description	Score	Coverage	Unique Peptides	Peptides	AA	MW kDa	Calc. pI
XP_027903254.1	serpinEZX [*Vigna unguiculata*]	54,39	48,35	2	16	42	46,9	7,64
XP_027916766.1	subtilisin inhibitor 1 [*Vigna unguiculata*]	9,36	43,88	2	2	98	11,1	5,01
XP_027922998.1	BowmanEBirk type seed trypsin and chymotrypsin inhibitorElike [*Vigna unguiculata*]	5,57	18,42	1	1	11	12,4	5,22
XP_027917589.1	heat shock 70 kDa protein [*Vigna unguiculata*]	38,29	7,4	3	3	64	71,1	5,39
NP_001304197.1	heat shock cognate 70 kDa protein 2Elike [*Vigna radiata*]	17,40	4,17	2	2	64	71,2	5,25

Table 1. List of the proteins present exclusively in P1 + P2 fractions. The protein composition of all the fractions was determined by a shotgun MS/MS strategy. The Table reports the proteins identified exclusively in P1 and P2 fractions upon differential analysis between P1 plus P2 and P3 mass spectrometry data. AA = number of amino acids, MW = molecular weight.

Among the different proteins identified, the Bowman-Birk domain trypsin and chymotrypsin inhibitor (BBI) was identified. Many studies are available concerning this family of peptides isolated from pulses (especially lentils and soybean) showing beneficial effects in CRC prevention[47,56,57]. They are small homodimeric peptides of 107 amino acids characterized by high variability among and also within legume species. The most conserved feature is the presence of 4 disulphide bridges within each monomer responsible for their high stability during the digestive processes, so that they are able to resist to pH levels equal to 1.5 without modifications that may affect their bioactivity. This means that the majority of the peptides assumed is able to reach intact the intestine and the colon[47,58].

This study showed that the BBI is maintained after 1-hour boiling of beans before the extraction procedure. Thus, once cooked and eaten they are very likely to reach the colon, where they are able to carry out their chemopreventive function. A recent work studying the effect of purified BBI from *V. unguiculata* on different breast cancer cell lines showed the internalization of the peptide in cells (probably mediated by endocytosis, since no receptors or carrier proteins were found on the

cellular membrane to promote its internalization) and its ability to enable the proteasome 20S functionality, activating a series of processes within cells that lead to apoptosis[43]. BBI internalization could explain the cytotoxic effect against CRC non-responsive cell lines to cetuximab.

The main biological function of the Bowman Birk inhibitor peptides in plants is related to defense mechanisms against predation and parasites through the inhibition of the digestion of protein and peptides, preventing insects feeding[59,60]. The biological activity shown by cowpea BBI against many pests is well known, so that many staple species such as rice and tomato have been genetically modified by using this gene to improve their resistance[61,62]. This aspect sheds new light on the possibility of finding suitable cultivation strategies to enhance the nutraceutical value of cowpea beans e.g., cultivation without the use of pesticides that could be hypothesised to elicit the production of these peptides by plants, therefore coupling healthy nutrition to environmental sustainability.

Conclusions

Legumes are fundamental raw food items supporting human diet, not only as a source of macronutrients such as proteins, starch, fibers and micronutrients (amino acids, vitamins, minerals), but also for their bioactive molecules (mainly polyphenols and peptides) able to provide benefits to human health. Our study

focused on one minor African species, *V. unguiculata*, not only for its importance for the economy and the sustenance of African population, but also for its adaptability and ease of cultivation. The results showed a chemopreventive action of *V. unguiculata* beans extract against different colorectal cancer cell lines, without affecting the healthy cell line and its ability to reduce cetuximab dose in colon cancer therapy. Based on the proteomic characterization and according to the literature the Bowman-Birk serine-protease inhibitor is supposed to be the main active component. The ability of this peptide to resist against boiling and low pH levels (such in the stomach) increases the feasibility of considering legumes such as the cowpea as fundamental supporters to contrast many different forms of CRC in the dietary context.

Supplementary information

Fig. S1 CCD841 cells proliferation (see Fig. 2 for details).

Fig. S2 Dose-response curves of E705 (**A**) and DiFi (**B**) with drug alone and drug plus two concentrations of extract.

Fig. S3 ^{1}H-^{1}H-TOCSY (**A**) and ^{1}H-^{13}C HSQC (**B**) spectra of a total cowpea water boiled seed extract sample dissolved in H_2O:D_2O 9:1 at a final concentration of 10 mg/mL, 25 °C, 600 MHz.

Fig. S4 FPLC profile of the purified extract.

Electronic supplementary information (ESI) available.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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Supplementary information

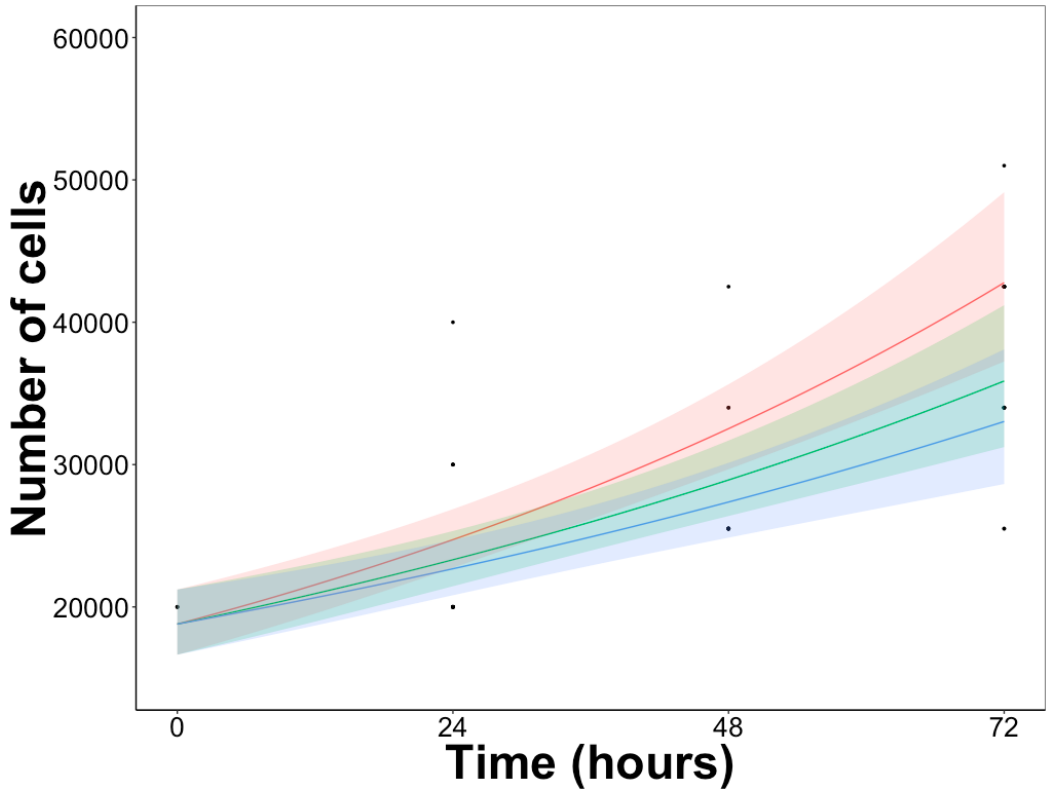

Fig. S1 CCD841 cells proliferation (see Fig. 2 for details).

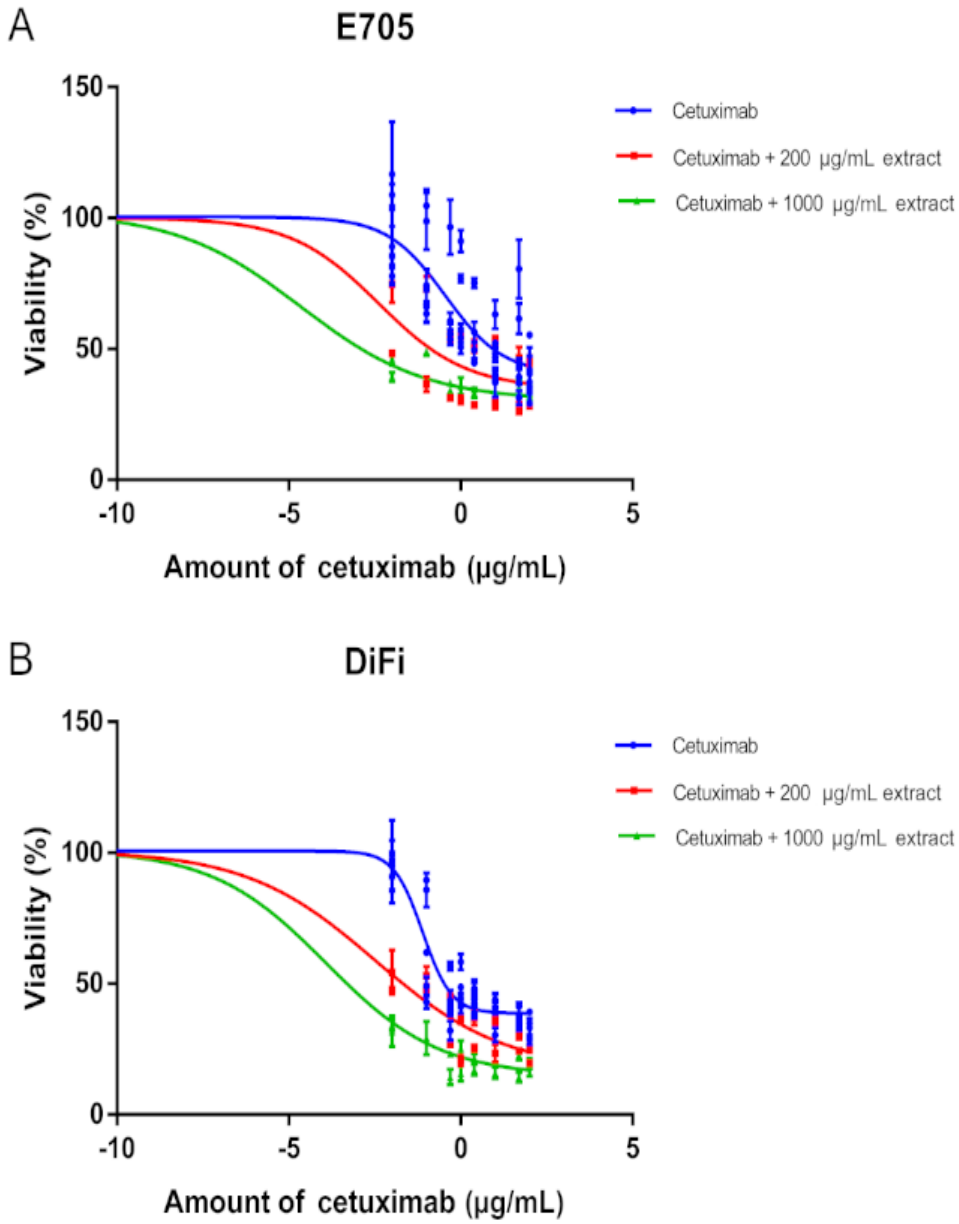

Fig. S2 Dose-response curves of E705 (A) and DiFi (B) with drug alone and drug plus two concentrations of extract.

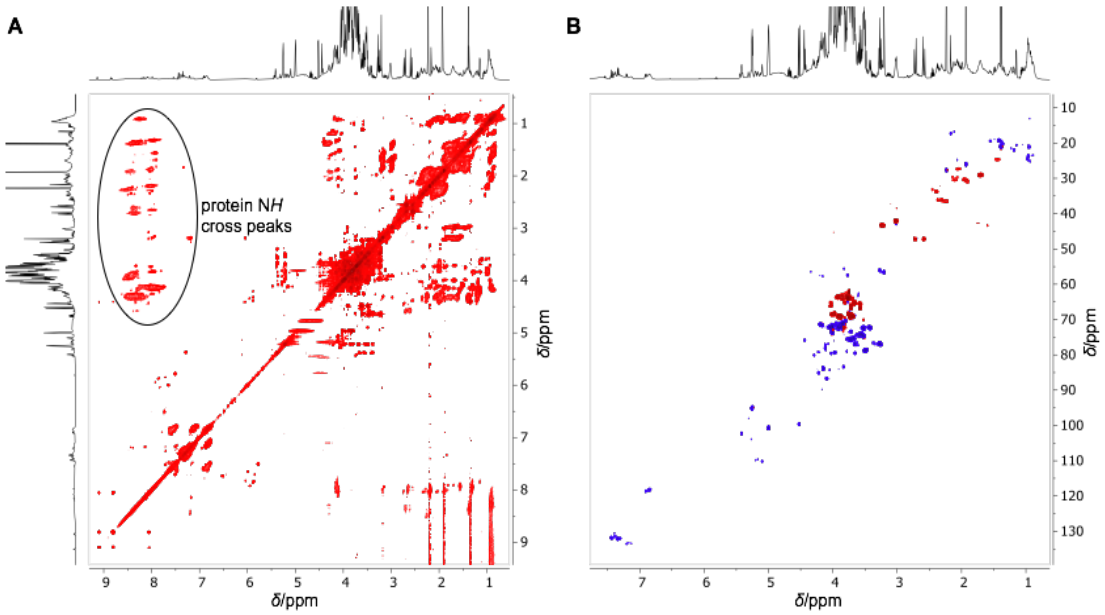

Fig. S3 ^{1}H-^{1}H-TOCSY (**A**) and ^{1}H-^{13}C HSQC (**B**) spectra of a total cowpea water boiled seed extract sample dissolved in H_2O:D_2O 9:1 at a final concentration of 10 mg/mL (TSP 0.5 mM, 25 °C).

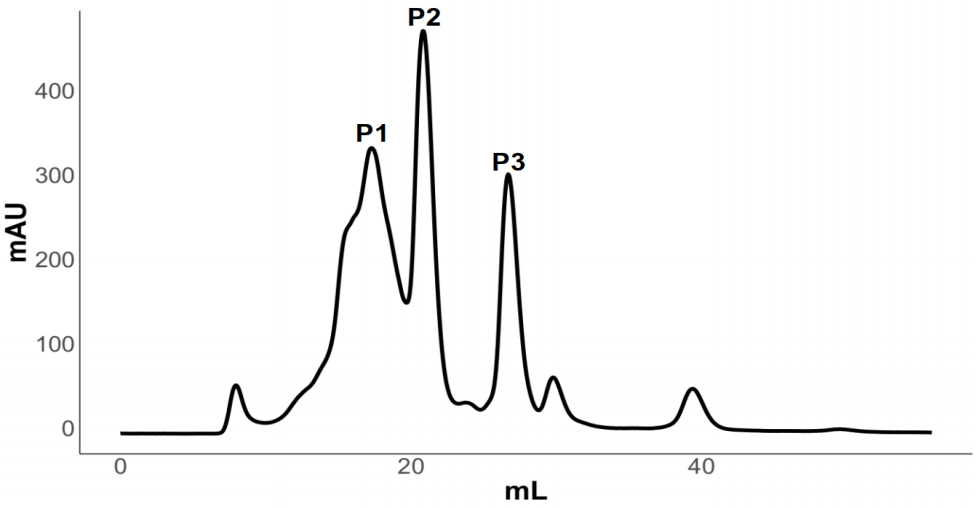

Fig. S4 FPLC profile of the purified extract.

4. Farida Tripodi, Linda Lombardi, Lorenzo Guzzetti, Davide Panzeri, Riccardo Milanesi, Manuela Leri, Monica Bucciantini, Cristina Angeloni, Daniela Beghelli, Silvana Hrelia, Giada Onorato, Elia Di Schiavi, Ermelinda Falletta, Simona Nonnis, Gabriella Tedeschi, Massimo Labra, Paola Coccetti (2020). Protective effect of *Vigna unguiculata* extract against aging and neurodegeneration. *Aging*, *12*.

In this work the ability of African pulses (originating from Arusha, Tanzania) to delay the outbreak of aging is assessed by using yeast cellular models. The bioactivity of cowpea seeds is widely deepened by using both *in vitro* and *in vivo* models of neurodegeneration, such as *Saccharomyces cerevisiae*, *Caenorhabditis elegans* and *Drosophila melanogaster* as well as human neuronal cell lines.

General considerations: Cowpea confirms to be a functional food because of its ability to delay aging and to reduce the impact of neurodegenerative disorders in several models. These evidences suggest that it may be a very interesting food ingredient in the elder population thanks to the many different preventive properties it exerts against the outbreak of age-related pathologies.

Protective effect of *Vigna unguiculata* extract against aging and neurodegeneration

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Keywords: *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, human α-synuclein, Parkinson's disease (PD)

ABSTRACT

Aging and age-related neurodegeneration are among the major challenges in modern medicine because of the progressive increase in the number of elderly in the world population. Nutrition, which has important long-term consequences for health, is an important way to prevent diseases and achieve healthy aging. The beneficial effects of *Vigna unguiculata* on metabolic disorders have been widely documented. Here, we show that an aqueous extract of *V. unguiculata* beans delays senescence both in *Saccharomyces cerevisiae* and *Drosophila melanogaster*, in a Snf1/AMPK-dependent manner. Consistently, an increased expression of FOXO, SIRT1, NOTCH and heme oxygenase (HO) genes, already known to be required for the longevity extension in *D. melanogaster*, is also shown. Preventing α-synuclein self-assembly is one of the most promising approaches for the treatment of Parkinson's disease (PD), for which aging is a risk factor. *In vitro* aggregation of α-synuclein, its toxicity and membrane localization in yeast and neuroblastoma cells are strongly decreased in the presence of bean extract. In a *Caenorhabditis elegans* model of PD, *V. unguiculata* extract substantially reduces the number of the age-dependent degeneration of the cephalic dopaminergic neurons. Our findings support the role of *V. unguiculata* beans as a functional food in age-related disorders.

INTRODUCTION

Nutrients and their metabolites control energy balance, enzymatic activities and genome stability throughout the lifecycle. It is an unequivocal statement that nutritional deficiency as well as excess contribute to the aging process. Dietary restriction is known as the most effective longevity intervention ranging from yeast to primates [1–6]. Several results have suggested new roles of key nutrients in the protection against aging and age-related disorders [5,7]. Thus, there is an increasing interest in nutrition as a way both to prevent diseases and to reach healthy aging.

Much of the current knowledge on the molecular mechanisms of aging comes from lifespan studies on short-lived model organisms, such as the budding yeast *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans* [8]. Specifically, AMPK (AMP-activated protein kinase), IGF (insulin-like growth factor) and TORC1 (target of rapamycin kinase complex 1) signaling pathways play key functions in regulating aging [9–11].

Neurodegenerative diseases, characterized by aberrant aggregates of the presynaptic protein α-synuclein, are collectively referred to as synucleinopathies, the second most common group of neurodegenerative diseases [12–14]. One of the most common synucleinopathies is Parkinson's disease (PD) and autosomal dominant forms of PD have been linked to mutations in α-synuclein. In PD patients, neurodegeneration is found predominantly in dopaminergic neurons. Despite the advances in the study of these pathologies, the detailed molecular mechanism of neuronal degeneration is still largely unknown. Several studies underline the relevant role of cellular models for a better understanding of the molecular regulation of human pathologies [15]. As such, budding yeast has been extensively employed in models of synucleinopathies [16–19]. In addition, an age-related degeneration of dopaminergic neurons has been shown in wild-type *C. elegans*

[20]. Interestingly, neuronal and dendritic loss are accelerated and more severe when human α-synuclein is expressed in dopaminergic neurons in *C. elegans* [21].

Vigna unguiculata (L.) Walp. or cowpea is the most relevant *Vigna* species for human food. It is cultivated in tropical and subtropical zones of the world, including Africa, Asia, Latin America and also in some Mediterranean countries [22,23]. Cowpea seeds are a good source of proteins, which mainly consist of globulins (vicilins or 7S globulins) and, to a lesser extent, albumins, glutelins and prolamins [24]. From a nutritional point of view, there is a high ratio of essential-to-non-essential amino acids, which is over 50%, suggesting the potential capacity of cowpea to cover human nutritional requirements [25,26]. Moreover, bioactive peptides with antioxidant activity are successfully obtained from enzymatic proteolysis of cowpea proteins, indicating also its potentiality as a functional food [24]. In comparison with other legumes, cowpea has a low-fat content with high level of unsaturated fatty acids and is also characterized by a high proportion of carbohydrates (mainly dietary fibers and resistant starch) [24]. Apart from the relevant source of essential macronutrients, cowpea also constitutes an interesting source of micronutrients [27].

All these features, together with the presence of minerals (calcium, iron and zinc) and phytochemicals, such as phenolic compounds, are attracting the attention of consumers and researchers, also because of its beneficial properties for health, including anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory and anti-hypertensive properties [28].

The aim of the present study is to investigate whether *V. unguiculata* also has anti-aging and neuroprotective effects, exploiting different model organisms to address complementary aspects. We show that an aqueous extract from *V. unguiculata*

beans increases lifespan in yeast cells, being dependent on Snf1/AMPK (sucrose-non-fermenting/AMP-activated protein kinase) and Ras/PKA (Rat sarcoma/protein kinase A) pathways. Its pro-longevity feature is also confirmed on the multicellular organism *D. melanogaster*, which is consistent with the increased expression of AMPK-dependent genes associated with fly lifespan extension. Cowpea extract is able to significantly reduce the aggregation of α-synuclein *in vitro* and to attenuate its toxicity both in yeast and neuroblastoma cells. Remarkably, in a nematode model expressing human α-synuclein, the age-dependent degeneration of the dopaminergic neurons is strongly reduced in the presence of chronic treatments with *V. unguiculata* extract.

RESULTS

Vigna unguiculata extract extends lifespan in yeast cells

Considering the nutritional properties and positive effects for health of *Vigna unguiculata* [28], we investigated the composition of aqueous bean extracts from *V. unguiculata* in comparison with those obtained from *Cajanus cajan* L. and *Phaseolus vulgaris* L., originating from the Arusha area in Tanzania. *V. unguiculata* extract was characterized by a higher starch amount and less proteins compared to the extracts obtained from the other pulses, while the percentage of total amino acids was comparable among species (Figure 1A-C). We also confirmed that cowpea seeds are a good source of amino acids (included the essential ones), as well as of unsaturated fatty acids (more abundant in comparison with the other two beans), confirming its nutritionally desirable features (Table S1, S2) [24].

To explore if these differences could have an impact on the longevity of yeast cells, exponentially growing cells were treated with 0.2% of the extracts from *V.*

unguiculata, *C. cajan* or *P. vulgaris* beans and chronological lifespan was monitored by measuring the viability of the cultures throughout time. Although all the extracts increased longevity of yeast cells, the highest response was evident in the presence of *V. unguiculata* one, with a mean lifespan increasing from 3 days to about 9.5 days (Figure 1D, Table 1). On the basis of the above results, we decided to continue our analysis by using only the cowpea extract. A strong dose-response effect on yeast longevity was observed by increasing the concentration of *V. unguiculata* extract in the culture (from 0.2% to 0.5%), since it was able to extend the mean lifespan up to 16 days at the higher concentration (Figure 1E, Table 1). Its anti-aging properties were evident also when the extract was added to "aged" cells, *i.e.* after they had already entered the stationary phase (Figure 1F). Remarkably, cowpea extract not originating from Arusha maintained the same effect, letting us to suppose that the origin of the beans has no relevant impact on its anti-aging features (data not shown).

Cell growth was then monitored in the presence of the highest concentration of the extract. Although the growth rate in the presence of 0.5% extract showed only a minor increase in exponential phase, the final biomass of the population was more than doubled in comparison with the control (Figure 1G). On the other hand, the consumption of glucose in the media during the exponential phase of growth was strongly reduced (more than 50%), suggesting that the presence of either starch or proteins induced a decrease of glucose uptake from the medium (Figure 1H). However, the lower glucose consumption in the presence of the extract have no effect on the experimental determination of CLS, which starts after carbon source exhaustion.

Importantly, the anti-aging effect of the extract was synergistic with caloric restriction, one of the most effective non-genetic interventions known to promote lifespan extension in several model organisms [10] (Figure S1).

Overall, the data presented indicate that cowpea aqueous extract extends chronological aging in yeast cells.

To increase our knowledge on the composition of *V. unguiculata* extract we performed a proteomic analysis. Interestingly, we identified 174 proteins with a molecular weight ranging from 200 to less than 20 kDa, of which 10% are oxidoreductase, 12% are stress response proteins while 25% are still uncharacterized (Figure 2A-B).

The signaling pathways connected to longevity regulation are well known in yeast. Among them, the Snf1/AMPK (sucrose-non fermenting/AMP-activated protein kinase) and the autophagic pathways are anti-aging pathways, while the Ras2/PKA (Rat sarcoma/protein kinase A) and the TORC1 (target of rapamycin complex 1) pathways are pro-aging ones [8,9,29]. To identify through which of them the cowpea extract extended yeast chronological lifespan, we tested its effect on mutants bearing deletion in one of the aforementioned pathways (*snf1Δ*, *atg1Δ*, *ras2Δ*, *tor1Δ*) (Figure 2C-F, Table 2). The anti-aging effect of 0.2% cowpea extract was still evident in *tor1Δ* and *atg1Δ* strains (Figure 2C,E, Table 2), while it was strongly reduced in *ras2Δ* and *snf1Δ* mutants (Figure 2D,F, Table 2), indicating that the Ras/PKA and the Snf1/AMPK pathways are involved in mediating the anti-aging effect of cowpea extract in yeast cells.

Vigna unguiculata* extract supplementation extends lifespan in *Drosophila melanogaster

To investigate the pro-longevity effect of the cowpea extract also in a multicellular organism, female Canton S flies were lifelong supplemented with 0.2% or 0.5% *V. unguiculata* extract. A significant marked increase in mean lifespan was observed in flies supplemented with 0.2% bean extract in respect to controls (40.09±1.08 days *vs* 31.82±0.86 days; 25.99% increase), while mean lifespan of flies supplemented with 0.5% bean extract was comparable to that of control flies (Figure 3A). These data are only partially in agreement with the results obtained in yeast cells, where the 0.5% cowpea supplementation was more effective than the 0.2% one. Nevertheless, considering only the survivorship data obtained after a 3 weeks supplementation, a higher survival of flies supplemented with 0.5% in respect to both 0.2% supplement and control was observed (Figure S2A-B).

To verify whether the increase of the mean lifespan in the presence of 0.2% extract was due to bean extract supplementation itself and not to CR induced by bean extract off-flavor, the body weights of flies were recorded at 30 and 45 days. No differences in fly body weights were observed, suggesting an equal food uptake in control and supplemented groups (Figure 3B).

To better clarify, at a molecular level, the positive effect of 0.2% cowpea extract supplementation on *D. melanogaster* lifespan, the expression of genes involved in preserving cellular homeostasis and longevity was investigated. Flies were supplemented with cowpea extract for 30 or 45 days and the expression of genes involved in aging-related signaling pathways (SIRT1 -sirtuin 1-, FOXO -Forkhead box O- and NOTCH) and antioxidant defense systems (HO - heme oxygenase and TRXR - thioredoxin reductase) were measured (Figure 3C-F). Oxidative stress has been recognized to play a key role in aging [30]. The oxidative stress theory of aging speculates that the functional losses typical of elderly are associated with the

accumulation of structural impairments caused by the oxidative damage to macromolecules [31]. HO expression was significantly up-regulated by cowpea extract supplementation after both 30 and 45 days (Figure 3C), while TRXR was not influenced at all (data not shown), suggesting that *V. unguiculata* extract partially modulates the endogenous antioxidant defense system.

SIRT1, a member of the class III NAD^+-dependent histone deacetylases (HDACs) has been implicated in the extension of longevity in *D. melanogaster* [32]. SIRT1 expression was significantly up-regulated in flies supplemented with cowpea extract after both 30 and 45 days (Figure 3D). Remarkably, also NOTCH expression increased at both time points (Figure 3E), in accordance with findings showing that SIRT1 is a positive modulator of NOTCH [33]. FOXO is a fundamental transcriptional regulator of the insulin pathway modulating growth and proliferation and its increase has been associated with extension of flies lifespan [34]. Although FOXO expression after 30 days of supplementation with *V. unguiculata* was the same as in control flies, cowpea extract triggered a significant up-regulation of FOXO expression at 45 days (Figure 3F). In agreement with the gene expression, the level of FoxO and Sirt1 proteins increased in flies supplemented with cowpea extract (Figure 3G-I).

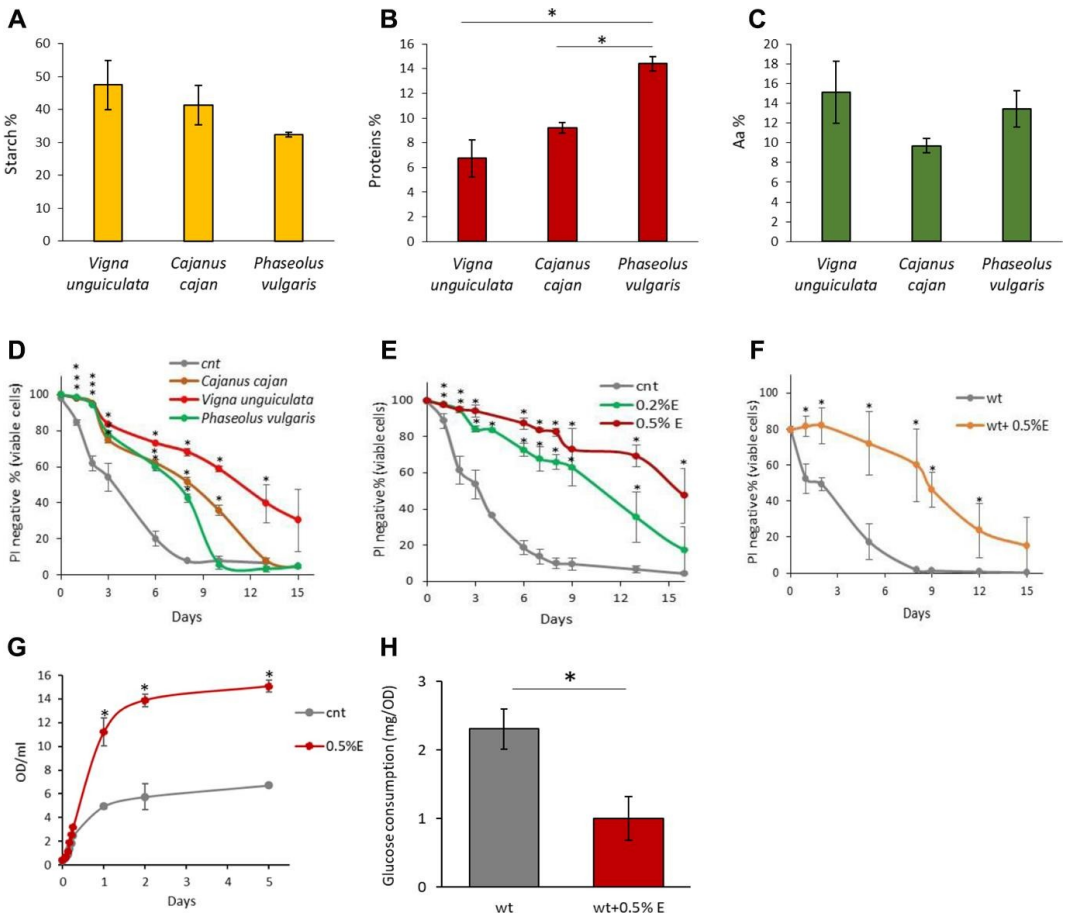

Figure 1. Chemical properties of bean extracts. (**A**) Starch content, (**B**) protein content and (**C**) amino acid content in *V. unguiculata*, *C. cajan* and *P. vulgaris* extracts. *p<0.05. (**D**) CLS of yeast cells grown in the absence or presence of 0.2% *V. unguiculata*, *C. cajan* and *P. vulgaris* extracts. *p<0.05 relative to control cells. (**E**) CLS of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.2% or 0.5% *V. unguiculata* extract, added in exponential phase of growth. *p<0.05 relative to control cells. (**F**) CLS of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.5% *V. unguiculata* extract, added to cells in stationary phase (and not in exponential phase, as in the other experiments). *p<0.05 relative to control cells. (**G**) Growth curves of yeast cells grown in SD medium containing 2% glucose in the absence or presence of 0.5% extract *V. unguiculata*. *p<0.05 relative to control cells. (**H**) Glucose consumption (mg/OD) of yeast cells grown in SD medium in the absence or presence of 0.5% *V. unguiculata* extract, measured on growth media sampled at multiple time points during exponential phase of growth (0.2-2.5 OD/ml). *p<0.05 relative to control cells.

wt strain	mean	maximal
cnt (no extract)	3.05 ± 0.42	9.46 ± 1.47
0.2% P. vulgaris	6.42 ± 0.12	11.72 ± 0.05
0.2% C. cajan	7.74 ± 0.30	13.53 ± 0.32
0.2% V. unguiculata	9.55 ± 0.49	18.55 ± 0.64
0.5% V. unguiculata	16.03 ± 3.01	>20

Table 1. Mean and maximal lifespan of wt cells grown in the presence of the indicated extracts.

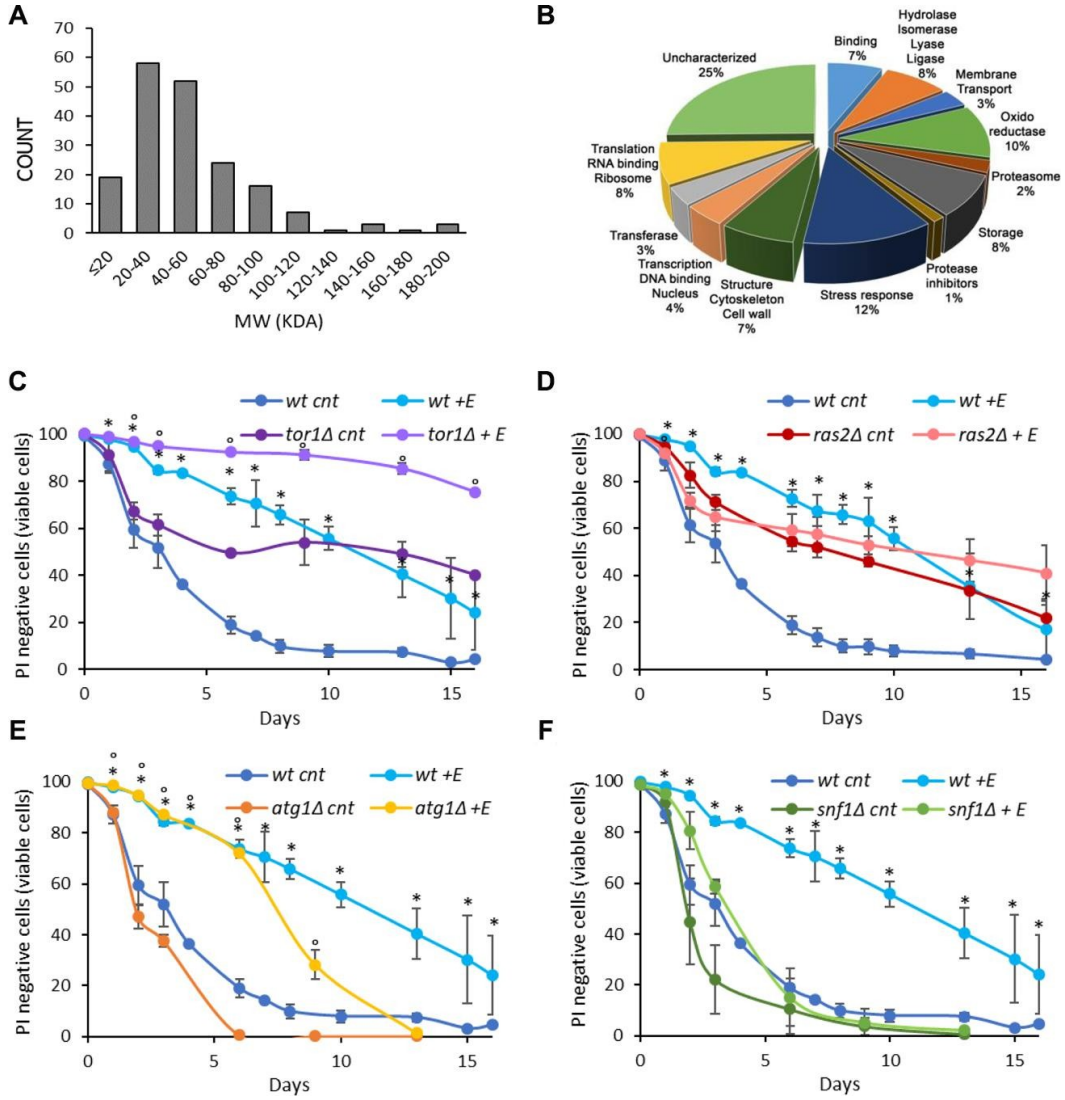

Figure 2. Cowpea extract extends yeast lifespan. (**A**, **B**) Analysis of *V. unguiculata* extract by mass spectrometry using a shotgun proteomic approach to identify all the proteins present in the sample. (A) MW distribution and (B) classification of the proteins identified. The data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017716. (**C**–**F**) CLS of wt and (**C**) *tor1Δ*, (**D**) *ras2Δ*, (**E**) *atg1Δ*, (**F**) *snf1Δ* cells, grown in SD medium containing 2% glucose in the absence or presence of 0.2% *V. unguiculata* extract. *p<0.05 relative to untreated wt cells, °p<0.05 relative to untreated mutant cells. Curves of wt untreated cells and treated with the extract were repeated in **C**–**F**.

	lifespan (days)			
	mean		maximal	
strain	cnt	0.2% E	cnt	0.2% E
wt	3.05 ± 0.42	9.55 ± 0.49	9.46 ± 1.47	18.55 ± 0.64
ras2Δ	7.48 ± 0.40	10.48 ± 4.98	18.95 ± 1.34	>20
tor1Δ	5.87 ± 0.23	>20	18.5 ± 0.14	>20
atg1Δ	2.22 ± 0.10	7.35 ± 0.21	4.95 ± 0.21	11.45 ± 0.78
snf1Δ	2.0 ± 0.42	3.35 ± 0.21	4.8 ± 2.40	7.2 ± 1.56

Table 2. Mean and maximal lifespan of mutant strains grown in the presence of 0.2% *V. unguiculata* extract. Data of wt cells were repeated for clarity.

Thus, the aging-related signaling pathways of SIRT1, FOXO and NOTCH are involved in mediating the effect of cowpea extract in fruit flies.

Vigna unguiculata extract reduces both α-synuclein toxicity and aggregation *in vitro*

Extensive literature reports the fibrillation-inhibiting effects of plant extracts, including those consumed as part of a healthy diet [35,36] and others found in traditional medicine [37–40].

α-Synuclein is a presynaptic protein associated with the pathophysiology of synucleinopathies, including Parkinson's disease [12–14], and budding yeast has been extensively employed in models of synucleinopathies [16]. Thus, the effect of *V. unguiculata* extract on the longevity of yeast cells over-expressing the human α-synuclein [41] was evaluated.

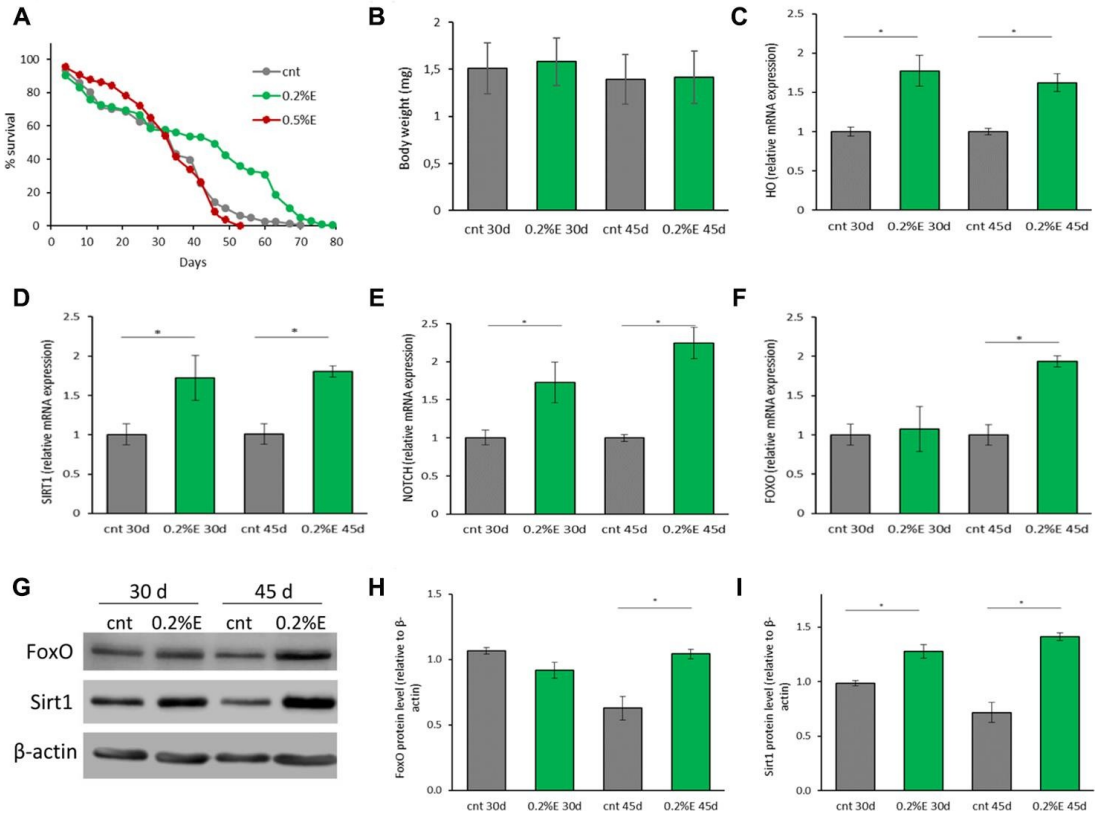

Figure 3. Cowpea extract extends *D. melanogaster* lifespan. (**A**) Survivorship of adult female *D. melanogaster*. Flies were supplemented with 0.2% and 0.5% bean extract lifelong. (**B**) Body weights of *D. melanogaster* supplemented with 0.2% bean extract. Flies were supplemented with 0.2% bean extract for 30 or 45 days. (**C**–**F**) Expression of genes related to longevity and oxidative stress. Flies were supplemented with 0.2% cowpea extract for 30 or 45 days. Total RNA was isolated and the mRNA levels of HO (**C**), SIRT1 (**D**), NOTCH (**E**), FOXO (**F**) were quantified using RT-PCR. (**G**) Western analysis using anti-FoxO and anti-Sirt1 antibodies on proteins extracts from flies supplemented with 0.2% cowpea extract for 30 or 45 days. (**H**–**I**) Densitometric analysis of FoxO and Sirt1 proteins. *p<0.05 with respect to the corresponding controls.

Interestingly, the addition of cowpea extract to exponentially growing cells strongly reduced the toxic effects of α-synuclein with a significant marked increase in mean lifespan (11.19 ± 2.18 days *vs* 2.22 ± 0.31 days; 404% increase) (Figure 4A, Table 3). Although α-synuclein protein was still present 3 days after the treatment (Figure S3), it was less localized to the plasma membrane, as shown by

immunofluorescence analysis (Figure 4B, C) and cell fractionation (Figure 4D). These data suggest that a different localization of α-synuclein, rather than its protein clearance, could be responsible for the reduced toxicity in the presence of the bean extract.

The process of α-synuclein fibrillation was then investigated *in vitro* at two different concentrations of cowpea extract. The increase of ThT and ANS fluorescence emission intensity was used to quantify fibrils formation and conformational change of the protein with or without cowpea extract (Figure 4E-F). The presence of *V. unguiculata* extract led to a significant concentration-independent decrease of ThT fluorescence in the α-synuclein aggregation solution, with an increase of the lag time and a decrease of both β-sheet growth rate and final equilibrium levels (Figure 4E). In agreement with a nucleation-dependent polymerization model, α-synuclein exhibited a sigmoidal binding without cowpea extract (Figure 4E).

These evidences suggest that *V. unguiculata* extract significantly altered the amyloid aggregation pattern of α-synuclein. Moreover, the ANS binding fluorescence data indicate that the cowpea extract might increase the formation of α-synuclein species with minor solvent exposure of hydrophobic clusters, or it might decrease the binding of ANS to α-synuclein surfaces (Figure 4F). The morphology of α-synuclein aggregates was also studied by TEM analysis. After 24 h of aggregation, the protein, either alone or in the presence of cowpea extract, existed as globular micelle-like and prefibrillar assemblies (Figure 4G). After 72 h of aggregation in the absence of the extract, α-synuclein samples were mostly mature fibrils. Remarkably, the presence of cowpea extract enriched the samples with short fibrils covered by densely packed globular clusters (Figure 4G). Overall, these

findings are consistent with an inhibitory effect of *V. unguiculata* extract on the formation of amyloid fibrils.

It has been reported that cytotoxicity of amyloidogenic species largely depends on their biophysical surface properties, which influences their reactivity with the cellular plasma membrane [42–44]. To assess α-synuclein toxicity, we performed MTT assays on the human neuroblastoma SH-SY5Y cell line exposed for 48 h to extracellular α-synuclein aggregates pre-formed *in vitro* in the absence or in the presence of cowpea extract. Coherently, α-synuclein obtained without extract supplementation exhibited the highest cytotoxicity: oligomers (Ol) and fibrils (Fib) showed about 70% and 50% viability, respectively (Figure 5A). In cells incubated with α-synuclein aggregates formed in the presence of the extract, toxicity was reduced and cell viability was about 83% with oligomers (Ol/E0.5) and 86% with fibrils (Fib/E0.5) increasing up to 100% and 95% at the highest concentration of the extract (Figure 5A). Accordingly, ROS level significantly decreased in cells exposed to oligomers and fibrils formed in the presence of cowpea extract (Figure 5B).

These data suggest that in the presence of *V. unguiculata* extract, α-synuclein aggregation is redirected into non-toxic aggregate species.

To further explore the potential mechanism for the protective effect of the cowpea extract, the interaction of α-synuclein aggregates with the plasma membrane of neuroblastoma cells was monitored by confocal microscopy. As previously reported [43,44], a large number of α-synuclein oligomers or fibrils (stained in red) were bound to the cell membrane (stained in green) (Figure 5C). When cells were exposed to α-synuclein aggregates formed in the presence of cowpea extracts, the binding of both oligomers and fibrils to the cellular membranes was drastically reduced (Figure 5C).

In conclusion, these data show that the presence of cowpea extract during α-synuclein aggregation decreases the ability of the resulting aggregates to bind the plasma membrane and to raise ROS production and cytotoxicity.

Vigna unguiculata* extract reduces α-synuclein induced neurodegeneration in *Caenorhabditis elegans

In order to evaluate the neuroprotective effects of cowpea extract on a multicellular organism, we turned to the nematode *C. elegans.* The expression of human α-synuclein in *C. elegans* causes the age-dependent degeneration and death of the four cephalic dopaminergic neurons (CEP), a phenotype which can be easily scored using a red fluorescent marker expressed only in those neurons [45]. Consistent with previous reports, we observed an age-related decline in the number of fluorescent dopaminergic neurons expressing human α-synuclein (Figure S4). Thus, we investigated the effects of *V. unguiculata* extract both at 0.2% and 0.5%, in 6-day adult animals (Figure 6A-B). While in mock treated animals a mean of 3 out of 4 CEP neurons died, in animals exposed to *V. unguiculata* extracts there was a partial rescue of neurodegeneration, with 2 neurons dying in 0.2% extract and only 1 in 0.5%. A similar effect was observed in animals treated with 3 mM valproic acid (positive control) [46].

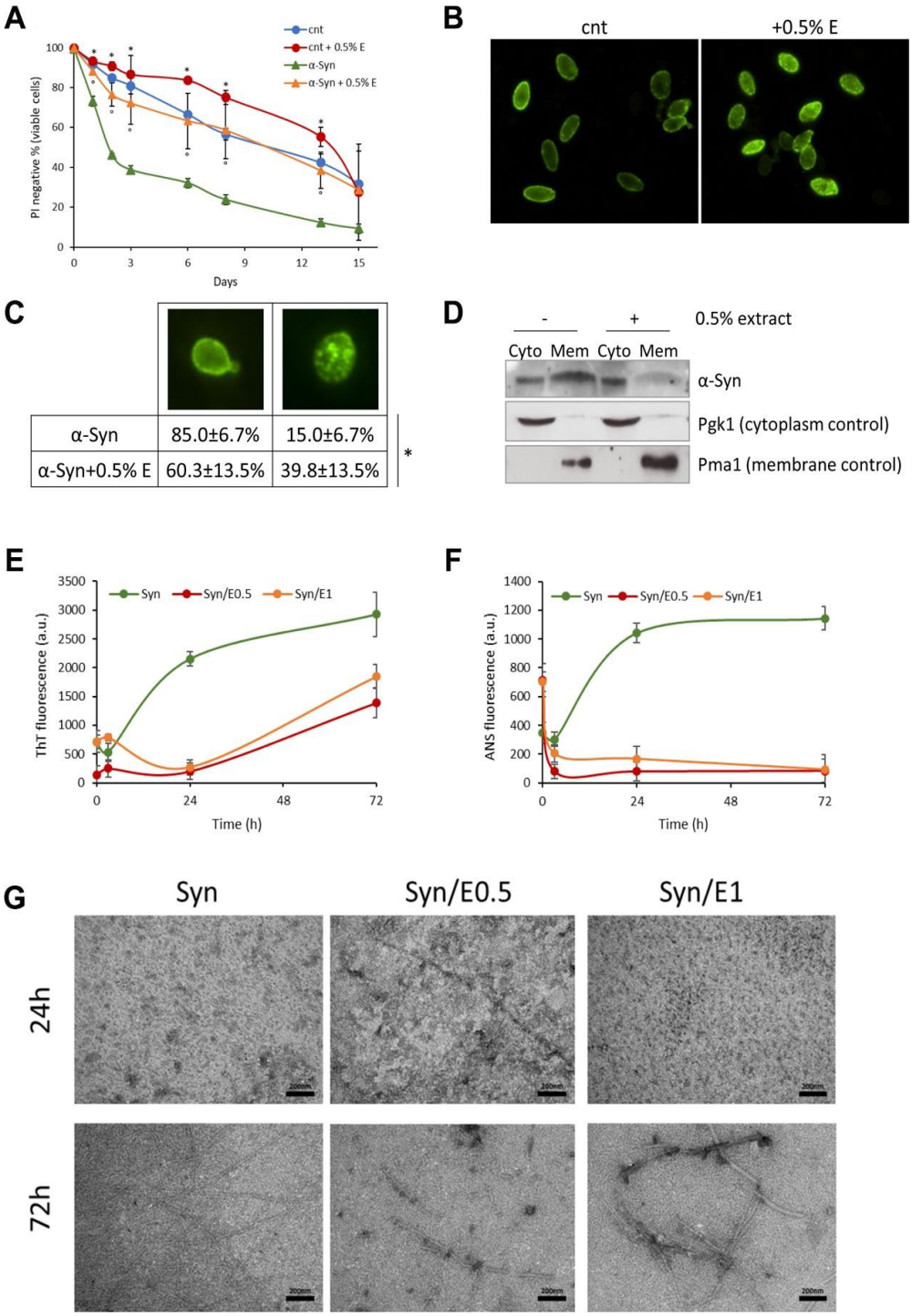
A
cnt
cnt + 0.5% E
α-Syn
α-Syn + 0.5% E
PI negative % (viable cells)
Days
B
cnt
+0.5% E
C
α-Syn
85.0±6.7%
15.0±6.7%
α-Syn+0.5% E
60.3±13.5%
39.8±13.5%
*
D
-
+
0.5% extract
Cyto Mem Cyto Mem
α-Syn
Pgk1 (cytoplasm control)
Pma1 (membrane control)
E
Syn
Syn/E0.5
Syn/E1
ThT fluorescence (a.u.)
Time (h)
F
Syn
Syn/E0.5
Syn/E1
ANS fluorescence (a.u.)
Time (h)
G
Syn
Syn/E0.5
Syn/E1
24h
72h

Figure 4. Cowpea extract reduces α-synuclein toxicity and aggregation. (**A**) CLS of yeast cells bearing pYX242 empty vector or pYX242-SNCA plasmid grown in SD medium containing 2% glucose in the absence or presence of 0.5% *V. unguiculata* extract. *p<0.05 relative to untreated cells bearing the empty vector, °p<0.05 relative to untreated α-synuclein expressing cells. (**B**, **C**) Immunofluorescence showing localization of α-synuclein in cells untreated or treated for 1 day with 0.5% *V. unguiculata* extract. The percentage of cells with α- synuclein localized in the cellular membrane is shown in (**C**). *p<0.05. (**D**) Western analysis using anti-α-synuclein antibody on cytolpasmic and membrane fractions isolated from wt [pYX242-SNCA] cells after 1-day treatment with 0.5% *V. unguiculata* extract. Pgk1 was used as cytoplasmic marker, Pma1 as membrane marker. (**E**, **F**) α-synuclein aggregation process followed by ThT fluorescence (**E**) and ANS binding (**F**) assays. (**G**) TEM pictures taken from α-synuclein aggregation mixture after 24 h and 72 h of incubation in the absence or in the presence of cowpea extract at molar ratio protein:extract 1:0.5 (E0.5) and 1:1 (E1); scale bars are shown.

	lifespan (days)			
	mean		maximal	
strain	**cnt**	**0.5% E**	**cnt**	**0.5% E**
[pYX242]	9.96 ± 0.49	13.3 ± 1.13	>15	>15
[pYX242-SNCA]	2.22 ± 0.31	11.19 ± 2.18	14.21 ± 1.06	>15

Table 3. Mean and maximal lifespan of yeast cells bearing pYX242 empty vector or pYX242-SNCA plasmid grown in the absence or presence of 0.5% *V. unguiculata* extract.

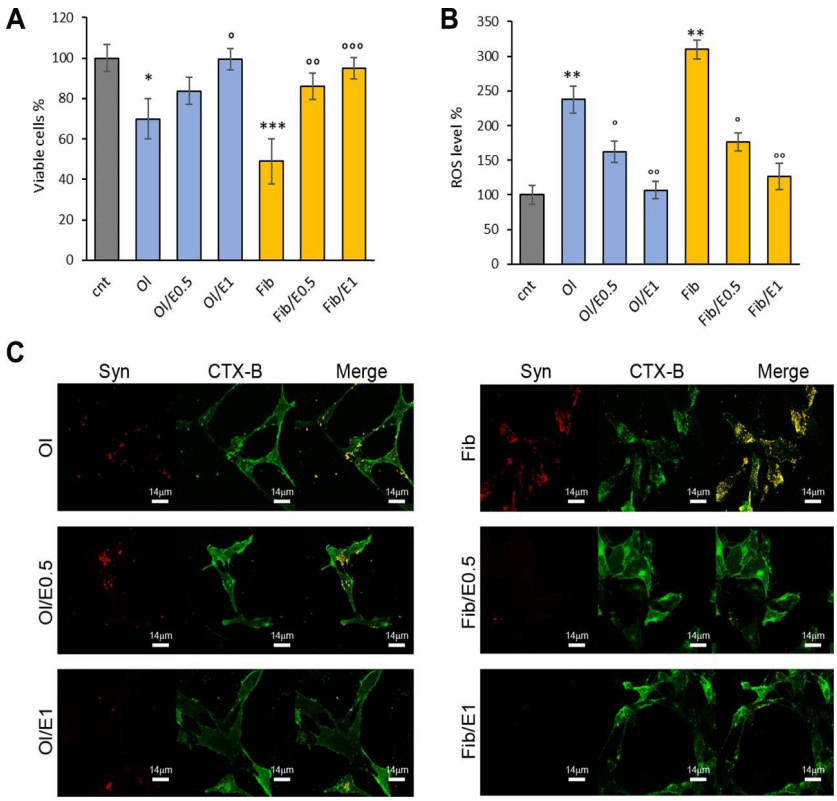

Figure 5. *V. unguiculata* extract reduces α-synuclein toxicity in neuroblastoma cells. (**A**, **B**) SH-SY5Y cells were grown for 48 h in the absence (cnt) or presence of 5 µM α-synuclein solution obtained after 24 h (oligomers, Ol) and 72 h (fibrills, Fib) of aggregation, without or with extract at molar ratio protein:extract 1:0.5 (E0.5) and 1:1 (E1). (**A**) Cell viability assessed by MTT assay and (**B**) ROS level evaluated by DCFDA fluorescence intensity assays. *p<0.05; **p<0.01; ***p<0.001 *vs* untreated cells. °p<0.05; °°p<0.01 *vs* treated cells with α-synuclein aggregates oligomeric (Ol) and fibrillar (Fib) grown without extract. (**C**) Z-projection of SH-SY5Y cell images by α-synuclein immunostaining (red) and CTX-B plasma membrane staining (green). Scale bars are shown.

Our results indicate that *V. unguiculata* extract protects CEP dopaminergic neurons from degeneration in a *C. elegans* model of α-synuclein toxicity, in a dose-dependent manner.

DISCUSSION

Cowpea is considered as a source of health-promoting compounds, with a low fat and high protein content, as well as dietary fibers, phenolic compounds and minerals. Consumption of cowpea is associated with reduced risk of gastrointestinal disorders, cardiovascular diseases, hypercholesterolemia, obesity, diabetes and several types of cancer [47]. We now add new important health benefits of cowpea beans, *i.e*. their anti-aging and neuroprotective effects. Indeed, we show that *V. unguiculata* extract extends lifespan, in two different eukaryotic models, such as budding yeast and fruit flies (Figures 1, 3). The extension of longevity requires Snf1/AMPK pathway in yeast (Figure 2F) and induces the upregulation of two downstream proteins of the AMPK pathway, such as FOXO and SIRT1 in *Drosophila* (Figure 3) [48-50]. Strikingly, it has been reported that AMPK and SIRT1 are downregulated with aging and their pharmacological activation is necessary to increase longevity [51].

The anti-senescence properties of cowpea extract are strongly additive with caloric restriction (Figure S1), the most effective non-genetic intervention delaying senescence [52], suggesting that *V. unguiculata* beans could display their best effect in terms of aging delay in a proper dietary regimen.

The strong neuroprotective features of cowpea extract are conserved in evolutionary distant eukaryotic systems. Indeed, *V. unguiculata* extract decreases α-synuclein toxicity in both yeast and neuroblastoma cells, as well as in a *C. elegans* PD model by partially rescuing the degeneration of cephalic dopaminergic neurons (Figure 6).

The anti-aggregation properties of cowpea on α-synuclein is clearly evident (Figure 4E-G). Along similar lines, the extract decreases the localization of α-synuclein to

cell membrane both in the yeast model, in which α-synuclein is intracellularly expressed (Figure 4A-D), and in neuroblastoma cells, where α-synuclein is added to the medium (Figure 5), also in keeping with the minor solvent exposure of hydrophobic clusters detected by ANS on amyloid assemblies (Figure 4F). These results suggest that *V. unguiculata* extract decreases the neurotoxicity caused by the intracellular accumulation of α-synuclein aggregates and the cellular damage induced by oligomeric aggregates interacting with the cell membrane by displacing the toxic protein from the lipidic bilayer. Our data are in accordance with recent results showing that inhibition of α-synuclein binding to membranes reduces the toxicity of the protein both in worms and in mice [53,54].

The aqueous extract of cowpea beans contains starch, amino acids, as well as several different proteins and peptides (Figure 1A-C, 2A,B), while it is probably very poor in phenolic compounds. Although abundance of starch and proteins is generally considered negative from an aging point of view [10], the protection against senescence and neurodegeneration might be the result of a synergistic effect of different elements. Indeed, the nutrient combination of the extract rather than a single component might be responsible for the metabolic reprogramming, which leads to the longevity phenotype.

Although the identity of the active components in the extract remains to be investigated, the extraction process strongly mimics the way in which these beans are consumed. Therefore, the anti-aging and neuro-protecting compounds are likely to be conserved during the cooking process. Remarkably, it has been reported that cooking legumes in water increases the insoluble fiber content, protein quality and digestibility, although with a reduction of the content of vitamins and minerals [47]. Therefore, the use of cowpea beans should be encouraged and eventually the

identification of the bioactive compounds could lead to the development of specific dietary supplements to support healthy aging and to delay neurodegeneration.

Any dietary intervention that has the potential of delaying the progression of age-related diseases could improve the quality of life of the aging population, inducing also an important impact on the economic implications of elderly on the society. Thus, *V. unguiculata* consumption in the global food chain is encouraging since our study suggests that cowpea beans supplementation can prevent age-related disorders.

Although data on bioactive compounds from cowpea are still poor, some reports indicate components like peptides may contribute to health benefits derived from cowpea [55]. Remarkably, several proteins identified by proteomic analysis are still uncharacterized (Figure 2A-B). We believe that additional work is necessary to discover the bioactive compounds in cowpea and their interactions to efficiently exploit them in foods, such as snacks and breakfast cereals by targeting benefits to immune function and health gut. Indeed, interesting data show that progression of PD has been frequently associated with dysbiosis of gut microbiota [56,57].

In conclusion, considering the role of functional food in the management of age-related diseases, we strongly support the intake of *V. unguiculata* beans to reduce senescence, neuroinflammation and the extent of neurodegeneration.

MATERIALS AND METHODS

Extract preparation. *V. unguiculata*, *C. cajan* and *P. vulgaris* seeds were purchased from two markets (Kilombero and Arusha Central Market) in Arusha, Tanzania (3°22'0.01"S, 36°40'59.99"E). Seeds of each species were boiled for 1 h and left cool down for the subsequent hour. The treatment was performed to mimic the

condition of consumption, as described in [58]. Then, seeds were incubated at 50°C overnight till dryness and grinded to obtain a fine powder. 2 g of seed dry powder were suspended in 50 ml of ultrapure MilliQ water. Then, pulses were extracted through a magnetic stirrer at 500 rpm for 5 minutes and centrifuged at 5000 g for 30 min. Supernatant was recovered and freeze-dried.

Chemical and proteomic characterization of the extracts.

Starch content. Starch content was evaluated by the enzymatic assay Total Starch AOAC Method 996.1 1 and AACC Method 76.13 (Megazyme ®, Ireland). Briefly, 50 mg of extract were suspended in 200 µl of ethanol 80% v/v and 1 ml of 2 M KOH. Samples were magnetically stirred for 20 min at 4°C. Then, 4 ml of sodium acetate pH=3.8 were added, followed by the addition of 50 µl of α-amylase (8300 U/mL) and 50 µl of amyloglucosidase (AMG, 3300 U/ml). Samples were incubated for 30 min with intermittent mixing on a vortex mixer, then centrifuged for 10 min at 3000 rpm to recover the supernatant. In order to evaluate total starch content, a reaction mixture was prepared as follows in a quartz cell: 1 ml H_2O, 25 µl of sample, 50 µl of a buffer solution pH = 7.6, 50 µl NADP+/ATP. The solution was incubated for 3 min at room temperature and then the absorbance was read at 340 nm against the blank. Then, 10 µl of a solution containing hexokinase (HK) and glucose-6-phosphate-dehydrogenase (G6PDH) was added. After an incubation of 5 min at room temperature, the absorbance was read against the blank again at 340 nm. Data are expressed as g of starch per 100 g of extract.

Amino acids content. Amino acids were quantified through a HPLC-DAD method. A 1260 Infinity II LC System (Agilent, USA, 2018) was set up for the analysis. The calibration curve was made up using an amino acid mixed solution (Merck, Germany) in a concentration range between 0.078 mM and 1.25 mM. The column

was an Agilent Poroshell HPH C18 (100 x 4.6 mm, 2.7 μm) coupled with a guard column (AdvanceBio Oligo 4.6 x 5 mm, 2.7 μm) and it was kept at 40°C. Mobile phases were: A - 10 mM Na_2HPO_4 pH=8.2 and B - Acetonitrile:Methanol:Water 45:45:10. The elution program was the following (%B): 0-0.35 min 2%, 13.4 min 57%, 13.5 min 100%, 15.7 min 100%, 15.8 min 2%, 18 min end. Flow rate was constant at 1.5 ml/min. All solvents were HPLC grade, whereas the buffer, solutions and samples were pre-filtered with a 0.22 μm filter. OPA (o-Phthaldialdehyde reagent, Merck, Germany) was chosen as derivatizing agent acting as a fluorophore. Injection volume was 10 μl. The signal used to visualize the fluorescence was set at 338 nm bandwidth 10 nm with a reference wavelength of 390 nm bandwidth 20 nm. All data were displayed and analyzed on Agilent ChemStation software. Data are expressed as g of amino acids per 100 g of extract.

Proteins content. Total Protein Content (TPC) was evaluated by using the Bradford assay as follows: 1 ml of 50% Coomassie-Brilliant Blue Bradford reagent (ThermoFisher, USA) was incubated at room temperature with 2 μl of extract of known concentration for a minute. Absorbance was read against blank at 595 nm and fitted on a calibration curve made up with BSA (Bovine Serum Albumin) in a range between 0 and 6 mg/ml. Data are expressed as g of protein per 100 g of extract.

GC/MS analysis. Before the GC/MS analyses all samples were subjected to a derivatization process, as described below. About 5 mg of each sample were accurately weighed, suspended in 50 μl of 2wt% methoxylamine hydrochloride in pyridine and incubated for 90 min at 37°C. Then, 80 μl of MBDSTFA (N-methyl-N-ter-butyldimethylsilyl-trifluoroacetamide)+1% TBDMCS (tert-butyldimethylchlorosilane) were added and the samples were incubated at 60° C

for 30 min. After incubation at room temperature overnight, the samples were analyzed by using a ISQ™ QD Single Quadrupole GC-MS (Thermo Fisher) equipped with a VF-5ms (30 m x 0.25 mm i.d. x 0.25 µm; Agilent Technology). Injection volume: 1 µl. Oven program: 100° C for 2 min; then 6° C/min to 280° C for 15 min; Run Time 42 min. Helium was used as the gas carrier. SS Inlet: Mode Splitless. Inlet temperature: 280° C. Flow 1.0 ml/min. MS transfer line: 270° C. Ion source: 250° C. Ionization mode: electron impact: 70 eV. Acquisition mode: full scan. In order to compare the composition of the extracts, for each analyte identified by GC/MS a target ion (m/z) was extracted by the TIC and the corresponding area was calculated. Table S2 reports for each analyte the corresponding target ion used.

Proteomic analysis. The extract was reduced, derivatized and digested with trypsin (protein: protease ratio 20:1) as described in [59] before MS.MS analysis.

Peptides separation was achieved on a Thermo Easy-nLC 1000, with a linear gradient from 95% solvent A (2 % ACN, 0.1% formic acid) to 30% solvent B (80% acetonitrile, 0.1% formic acid) over 60 min, from 30 to 60% solvent B in 5 min and from 60 to 100% solvent B in 2 min at a constant flow rate of 0.25 µl/min, with a single run time of 75 min. MS data were acquired on a Thermo Q-Exactive-HF, with a data-dependent top 15 method, the survey full scan MS spectra (300-1650 m/z) were acquired in the Orbitrap with 60000 resolution, AGC target 3e6, IT 20 ms. For HCD spectra resolution was set to 15000, AGC target 1e5, IT 80 ms; normalized collision energy 28 and isolation width of 1.2 m/z.

Raw label-free MS/MS files from Thermo Xcalibur software (version 4.1) [59] were analyzed using Proteome Discoverer software (version 2.2, Thermo Fisher Scientific) and searched with Sequest algorithm against the proteome of NCBI Phaseoleae (release 05/08/2019). The minimum required peptide length was set to

6 amino acids with carbamidomethylation as fixed modification, Met oxidation and Arg/Gln deamidation as variable modifications.

The mass spectrometry proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE [60] partner repository with the dataset identifier PXD017716.

Yeast methods.

Yeast strains and media. The yeast strains used in this paper are listed in Table S3. Cells were grown at 30°C in minimal medium containing 2% glucose as a carbon source and 0.67% yeast nitrogen base without amino acids, supplemented with 50 mg/l of required amino acids and bases for which the strains were auxotrophic. The natural extracts were dissolved in the medium at a concentration of 0.2% or 0.5% and filtered through 0.22 μm filters.

Chronological lifespan experiments (CLS). Cell cultures were grown in liquid medium until mid-late exponential phase and then inoculated into flasks containing medium in the presence or absence of the natural extracts (0.2% or 0.5% as indicated in each experiment). Survival was assessed by propidium iodide staining (PI) at the indicated time points with the Cytoflex cytofluorimeter (Beckman Coulter) and analyzed with the Cytoflex software. For some experiments, survival was also confirmed by colony-forming units (CFUs) after 2 days of incubation at 30°C on YEPDA agar plates.

Protein extraction, cell fractionation and immunoblotting. Equal amounts of cells were collected and quenched using TCA 6% and lysed in lysis buffer (6M UREA, 1% SDS, 50 mM Tris-HCl pH7.5, 5 mM EDTA). The cytoplasmic-membrane fractionation experiment was conducted using the MEM-PER kit (Thermo), following the manufacturer's instructions on yeast spheroplasts. Western blot analysis was

performed using anti-Synuclein antibody (1:1000, Sigma Aldrich), anti-Pgk1 antibody (1:1000, Molecular Probes, used as loading control and cytoplasmic marker) and anti-Pma1 antibody (1:1500, Abcam, used as membrane marker).

Glucose consumption assay. Extracellular levels of glucose were evaluated on growth media of wt cells exponentially growing in the absence or presence of 0.5% cowpea extract, using the Megazyme D-glucose-HK assay kit, following the manufacturer's instructions, using an EnSight Plate Reader (Perkin Elmer).

Fluorescence microscopy on yeast. *In situ* immunofluorescence was performed on formaldehyde-fixed cells and carried using α-synuclein immunostaining (1:2000, Sigma Aldrich) followed by indirect immunofluorescence using rhodamine-conjugated anti-rabbit antibody (1:1000, Pierce Chemical Co). Digital images were taken with a Nikon DS-Qi MC camera mounted on a Nikon Eclipse 600 and controlled by the NIS elements imaging software (Nikon) with an oil 100X 0.5-1.3 PlanFluor oil objective (Nikon).

***In vitro* aggregation of α-synuclein.** α-synuclein was expressed in *Escherichia coli* BL21(DE3) cells transformed with the pET28b/α-synuclein plasmid. The recombinant protein was expressed and purified according to a previously described procedure [43] and further purified by RP-HPLC. The identity and purity of the eluted material were assessed by mass spectrometry**.** Protein samples (250 µM), filtered through a 0.22 µm pore-size filter (Millipore, Bedford, MA, USA) were incubated at 37°C in 20 mM sodium phosphate buffer, pH 7.4 up to 3 days under shaking at 900 rpm with a thermo-mixer in the absence or in the presence of extract by using molar protein/substance ratios of 1:0.5 (E0.5) and 1:1 (E1). Oligomer-enriched or fibril-enriched sample were prepared by incubating a-synuclein for 24 h or 72 h, respectively.

ThT assay. The ThT binding assay was performed according to LeVine [61], using a 25 µM ThT solution in 20 mM sodium phosphate buffer, pH 7.0. Each sample, diluted at a final concentration of 6.25 mM, was transferred into a 96-well half-area, low-binding, clear bottom (200 µl/well) and ThT fluorescence was read at the maximum intensity of fluorescence of 485 nm using a Biotek Synergy 1H plate reader; buffer fluorescence was subtracted from the fluorescence values of all samples. In controls experiments, a significant interference of the highest concentrations of cowpea extract on ThT fluorescence was observed, so the two molar ratio protein:extract with lowest fluorescence interference were selected (Figure S5).

ANS assay. Samples containing aggregating a-synuclein with and without cowpea extract at 250 µM were investigated for their ability to bind 8-anilinonaphthalene-1-sulfonic acid (ANS; Sigma Aldrich, Saint Louis, MO, US). 5 µl of each samples at different times of aggregation was transferred into a 96-well half-area, low-binding, clear bottom (200 µl/well), and ANS (50 mM) fluorescence intensity was read at the binding intensity of fluorescence of 480 nm in a Biotek Synergy 1H plate reader; buffer fluorescence was subtracted from the fluorescence values of all samples. In control experiments, a significant interference of the highest concentrations of the extract on ANS binding fluorescence was observed, so we selected the two molar ratios protein:extract with the lowest fluorescence interference (Figure S5).

Transmission electron microscopy (TEM) imaging. 5 µl aliquots of a-synuclein aggregated in the presence or in the absence of cowpea extract were withdrawn at different aggregation times, loaded onto a formvar/carbon-coated 400 mesh nickel grids (Agar Scientific, Stansted, UK) and negatively stained with 2.0% (w/v) uranyl

acetate (Sigma-Aldrich). The grid was air-dried and examined using a JEM 1010 transmission electron microscope at 80 kV excitation voltage.

Cell culture methods.

Cell culture. SH-SY5Y human neuroblastoma cells were cultured at 37 °C in complete medium (50% HAM, 50% DMEM, 10% fetal bovine serum, 3 mM glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin), in a humidified, 5% CO_2 incubator.

MTT assay. Cell viability was assessed by the MTT assay optimized for the SH-SY5Y cell line. Briefly, SH-SY5Y cells were seeded into 96-well plates at a density of 10000 cells/well in fresh complete medium and grown for 24 h. Then, cells were exposed for 48 h to 5 µM α-synuclein obtained at different times of aggregation in the presence or in the absence of the *Vigna unguiculata* extract. Cells were also treated with the corresponding concentrations of extract used in the aggregation of α-synuclein and the viability resulted similar to that of untreated control cells. After 48 h of incubation, the culture medium was removed and cells were incubated for 1 h at 37°C in 100 µl serum-free DMEM without phenol red, containing 0.5 mg/ml MTT. Then, 100 µl of cell lysis solution (20% SDS, 50% N,N-dimethylformamide) was added to each well and samples were incubated at 37°C for 2 h to allow complete cell lysis. Absorbance values were measured using iMARK microplate reader (Bio-Rad) at 595 nm. Final absorption values were calculated by averaging each sample in triplicate after blank subtraction. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA).

ROS determination. Intracellular reactive oxygen species (ROS) were determined using the fluorescent probe 2',7'–dichlorofluorescein diacetate, acetyl ester (CM-H2 DCFDA; Molecular Probes), a cell-permeant indicator for ROS that becomes

fluorescent upon removal of the acetate groups by cellular esterases and oxidation. SH-SY5Y cells were plated on 96-well plates at a density of 10000 cells/well and exposed for 48 h to the α-synuclein samples. Then, 10 μM DCFDA in DMEM without phenol red was added to each well. The fluorescence values at 538 nm were detected after 30 min by Fluoroscan Ascent FL (Thermo-Fisher). Cells were also treated with the corresponding concentrations of extract used in the aggregation of α-synuclein and ROS levels resulted similar to that of untreated control cells. Statistical analysis of the data was performed by using one-way analysis of variance (ANOVA).

Confocal imaging. Subconfluent SH-SY5Y cells grown on glass coverslips were exposed for 48 h to 5 μM (monomer concentration) α-synuclein aggregates grown in the presence or in the absence of cowpea extract at different molar ratios (1:0.5, E0.5; 1:1, E1). Cell membrane labelling was performed by incubating the cells with 10 ng/ml Alexa Fluor 488-conjugated CTX-B (Cholera toxin B-subunit) in cold complete medium for 30 min at room temperature. Then, cells were fixed in 2.0% buffered paraformaldehyde for 6 min and permeabilized by treatment with a 1:1 acetone/ethanol solution for 4 min at room temperature, washed with PBS and blocked with PBS containing 0.5% BSA and 0.2% gelatin. After incubation for 1 h at room temperature with rabbit anti-synuclein polyclonal antibody (1:600 in blocking solution), the cells were washed with PBS for 30 min under stirring and then incubated with Alexa Fluor 568-conjugated anti-rabbit secondary antibody (Molecular Probes) diluted 1:100 in PBS. Finally, cells were washed twice in PBS and once in distilled water to remove non-specifically bound antibodies. Digital images were taken with a confocal Leica TCS SP8 scanning microscope (Leica, Mannheim, Ge) equipped with a HeNe/Ar laser source for fluorescence measurements. The

observations were performed using a Leica HC PL Apo CS2 X63 oil immersion objective.

***Drosophila* methods.**

Fly husbandry and supplementation and longevity assay. Wild type *Drosophila melanogaster* (Canton S) was kindly provided by Dr Daniela Grifoni (University of Bologna, Italy). Flies were maintained at constant temperature (25°C) and humidity (60%) with a 12/12 h light–dark cycle. Flies were reared on Formula 4-24 ® media (Carolina Biological, Burlington, NC, USA). The composition of this diet, as indicated by the manufacturer, is as follows: oat flour, soy flour, wheat flour, other starches, dibasic calcium phosphate, calcium carbonate, citric acid, niocinamide, riboflavin, sodium chloride, sodium iron pyrophosphate, sucrose, thiamine, mononitrate, brewer's yeast, emulsifier preservatives, mold inhibitor, food coloring. The Formula 4-24 diet requires separate application of yeast pellets (Saccharomyces cerevisiae) and saturation of this dry media mixture with water. After eclosion, males and females emerged within 1-2 day were allowed to mate freely for two days before female separation into vials containing 1 g Formula 4-24 Instant Drosophila Medium (Carolina) soaked with 4 ml water containing 0.5% or 0.2% bean extract. A total of 20 flies were placed in each vial.

Female flies emerging within a 2-day period were collected under FlyNap (Carolina) anaesthesia. A total of 600 fruit flies were divided into 3 groups: control group, flies supplemented with 0.2% bean extract and flies supplemented with 0.5% bean extract. Flies were transferred into vials containing fresh food every 2-3 days and the number of living flies was counted. This was repeated until all flies had died. Kaplan-Meier survival curves were generated for lifespan assessment. Further experimental details are in Supporting information.

Measurement of *Drosophila* body weights. Changes in body weights were used as an indicator of the food intake. Flies were fed on standard diet with and without bean extract for 30 or 45 days. For each condition (0.2% bean extract supplementation and control), five vials containing 20 flies/vial were counted.
Flies in each group were anesthetized by FlyNap (Carolina) and then weighed on a balance. The mean body weights of the flies in each group were calculated.

Gene expression analysis. Total RNA was extracted from the whole bodies of either 30 days or 45 days old flies belonging to the 0.2% group by using RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). The 0.2% supplementation has been chosen because it was the one able to significantly increase lifespan in *Drosophila*. All the experiments were performed in triplicate. The yield and purity of the RNA were measured using NanoVue Spectrophotometer (GE Healthcare, Milano, Italy). Only samples with density ratios A260/A280>1.8 were used. cDNA was obtained by reverse transcribing mRNA starting from 1 µg of total RNA using iScript cDNA Synthesis Kit (BIO-RAD, Hercules, CA, USA), following the manufacturer's protocol. The subsequent polymerase chain reaction (PCR) was performed in a total volume of 10 µl containing 2.5 µl (12.5 ng) of cDNA, 5 µl SsoAdvanced Universal SYBR Green Supermix (BIO-RAD), 2 µl of dH20 RNA free and 0.5 µl (500 nM) of each primer. The primers used are reported in Table 3 and RPL32 was used as reference gene.

Protein extraction and immunoblotting. Proteins were isolated from the whole bodies of either 30- or 45-day old flies. Proteins were homogenized using lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 60 mM dithiothreitol (DTT), 0.002% bromophenol blue) and centrifuged at 12000 g at 4 °C for 5 min. The supernatant was collected and mixed with Sample Buffer, Laemmli 2× Concentrate (Sigma Aldrich). Proteins were then loaded onto 4-20% SDS-PAGE gels followed by transfer

onto nitrocellulose membranes and immunoblotted with appropriate antibodies. Anti-Sirt1 (1:1000; Cell Signaling Technology, Beverly, MA), anti-dFoxO (1:1000, Covalab, Villeurbanne, France) and anti-β-actin (1:2000, Invitrogen Carlsbad, CA, USA) antibodies were used as primary antibodies. The HRP-conjugated anti-mouse IgG and anti-rabbit IgG antibodies were employed as the secondary antibodies (1:10000; Cell Signaling Technology). Targeted proteins were visualized using Clarity™ Western ECL Substrate (BIO-RAD). Densitometric analysis of specific immunolabeled bands was performed using ImageJ software.

Statistical analysis. Each experiment was performed at least three times, and all values are represented as means ± SD. One-way ANOVA was used to compare differences among groups followed by Dunnett's (Prism 5; GraphPad Software, San Diego, CA). Values of $p<0.05$ were considered statistically significant. Survival curves were prepared by Kaplan-Meier survival analysis and analyzed using the OASIS2 software [62].

***C. elegans* methods.**

***C. elegans* strains and treatment with *V. unguiculata* extract.**

Standard procedures for *C. elegans* strain maintenance were followed [63]. The strain used in this study, JZF142 [*pdat-1::hαSyn; pdat-1::DsRed*], was kindly provided by Prof. J. Feng (Case Western Reserve University, US) [45]. The *C. elegans* strain was grown on Nematode Growth Medium (NGM) containing agar, seeded with *E.coli* OP50 at 20°C. Lyophilized extract from *V. unguiculata* was solubilized in sterile distilled water at two dilutions, 2% or 5% w/V, and sterilized with 0.22µm filter. α-synuclein expressing animals were exposed to the following treatments: 0.2% or 0.5% of *V. unguiculata* extract, water as negative control (mock) and 3 mM valproic acid (VA) as positive control [46]. *C. elegans* animals at L4 developmental

stage were transferred into 12-well plates with NGM agar containing the different conditions as quadruplicates and allowed to become adults and lay eggs. After 14 hours the adults were discarded and the synchronized F1 progeny was allowed to grow in the presence of chronic treatments. F1 animals have been transferred every 3 days on new plates with treatment, until the day of analysis, to maintain them well fed and separated from the next generation.

The morphology of the four cephalic dopaminergic neurons (CEP neurons) in the F1 treated animals was scored at 6 days post adult stage. The neurodegeneration analysis was performed also on untreated animals at young adult and at 6 days post-adult stage. Animals were mounted and anesthetized with 0.01% tetramisole hydrochloride on 4% agar pads. The neurodegeneration analysis was performed using Zeiss Axioskop microscope (Carl Zeiss). All images were obtained using a Leica SP2 confocal laser scanning microscope (Leica). The spectra used for imaging DsRed were: λ excitation=543 nm and λ emission=580-630 nm. GraphPad Prism software was used for statistical analysis. The statistical significance was determined using Mann Whitney test or One-way ANOVA with Kruskal–Wallis post-test. Data are reported as averages of multiple observations ± SEM.

AUTHOR CONTRIBUTIONS

L.G. and D.P. procured the matrixes, prepared and characterized the extracts. E.F. performed GC/MS analysis. S.N. and G.T. performed proteomic analysis on *V. unguiculata* extract. F.T., L.L., R.M. performed experiments in yeast cells. M.L. performed *in vitro* assays on α-synuclein and experiments on neuroblastoma cells.

D.B. performed experiments in *D. melanogaster*. G.O. performed experiments in *C. elegans*. M.B. conceived and provided materials for the experiments and participated to manuscript preparation. C.A., D.B. and S.H. designed experiments in *D. melanogaster* and participated to manuscript preparation. E.D.S. designed experiments in *C. elegans* and participated to manuscript preparation. M.L. got founding and participated to manuscript preparation. P.C. and F.T. designed experiments in yeast cells and wrote the paper. P.C. coordinated the project.

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CONFLICT OF INTEREST

The authors have declared no conflict of interests.

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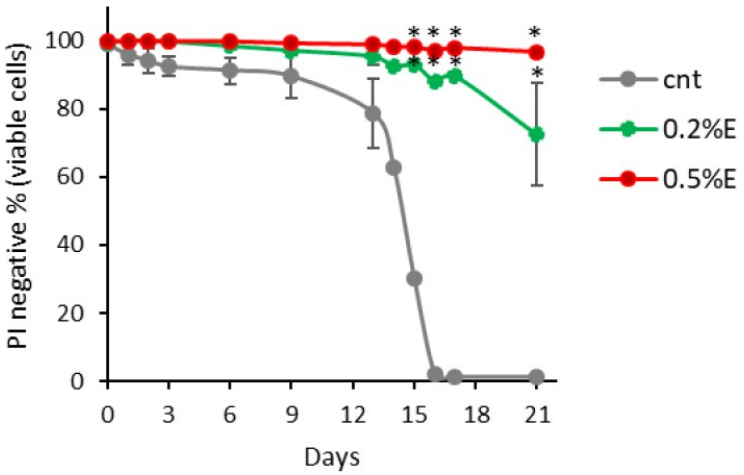
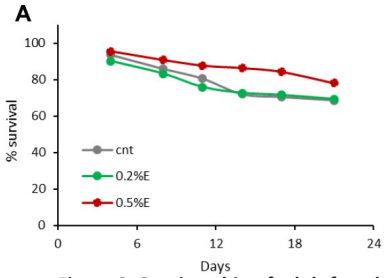
Supplementary material



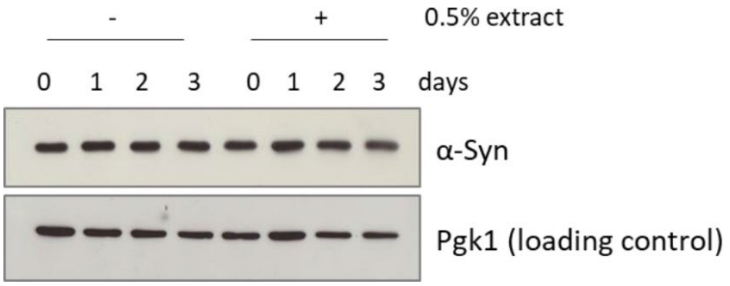
Supplementary Figure 1. The effect of cowpea extract is synergistic with caloric restriction. CLS of yeast wt cells grown in SD medium containing 0.5% glucose in the absence or presence of 0.2% or 0.5% extract of *Vigna unguiculata*. *p<0.05 relative to control cells.



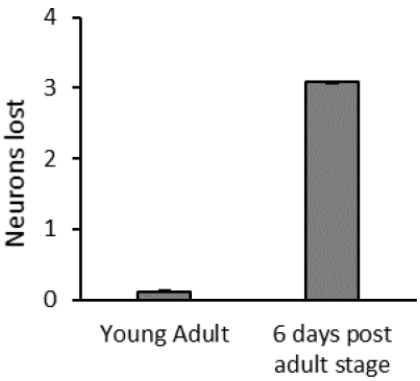
B

Condition	P-value
CTRL_F v.s. 0.5% bean_F	0.0032
CTRL_F v.s. 0.2% bean_F	0.9985
0.5% bean_F v.s. 0.2% bean_F	0.0032

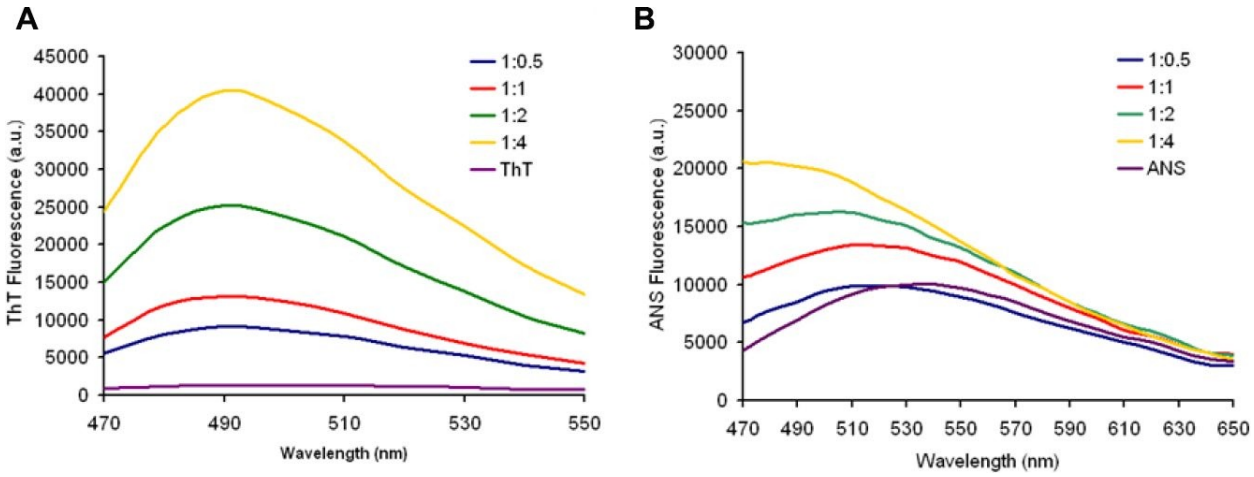
Supplementary Figure 2. Survivorship of adult female *D. melanogaster*. (**A**) Flies have been supplemented with 0.2 and 0.5% cowpea extract lifelong. Data are presented as percentage survival of flies as function of time (in days). (**B**) Analysis of the survivorship data with log-rank test using the online application for the survival analysis of life-span assays OASIS.



Supplementary Figure 3. α-synuclein level is unchanged but it is differently localized in the presence of cowpea extract in yeast cells. Western blot analysis is using anti-synuclein antibody in wt [pYX242-SNCA] cells after 5h, 1 day, 2 days, 3 days treatment with 0.5%*V. unguiculata* extract. Pgk1 was used as loading control.



Supplementary Figure 4. α-synuclein causes age-related dopaminergic neurons lost in *C.elegans*. Number of nonvisible CEP dopaminergic neurons in human α-synuclein expressing strain. In a wild type strain four CEP neurons expressing DsRed are always visible (not shown). In young adults almost no neurodegeneration is visible, while 6 days after adult stage a mean of 3 neurons is lost ($p<0.0001$ Mann- Whitney non parametric test). Error bars represent the SEM for two independent experiments. The number of young adult animals scored is 100, while after 6 days post adult stage is 91.



Supplementary Figure 5. Extract interferences on ThT and ANS fluorescence.

	Phaseolus vulgaris	Cajanus cajan	Vigna unguiculata
aa and bases			
glycine	1.00	1.07	2.66
L-leucine	1.00	0.49	2.90
L-threonine	1.00	0.00	10.74
L-alanine	1.00	0.80	2.72
L-proline	1.00	34.35	3.94
L-asparagine	1.00	0.63	52.44
L-aspartic acid	1.00	1.55	1.05
hypoxanthine	absent	absent	present
D-pyroglutamic acid	1.00	3.93	3.17
methylthiouracil	1.00	1.11	2.40
glycolysis/fermentation and TCA			
citric acid	1.00	1.90	2.25
malic acid	1.00	0.11	0.17
lactic acid	1.00	0.98	4.60
fatty acids			
glycerol	1.00	0.71	3.28
linolealidic acid	1.00	5.33	8.41
linolenic acid	1.000	0.162	2.663
stearic acid	1.000	0.968	1.342
palmitic acid	1.000	0.779	1.209
other organic acids			

D-pipecolic acid	1.00	0.78	absent
2-pentenedioic acid	1.00	1.34	1.14
2-thiobarbituric acid	1.00	0.66	0.29
L-dihydroorotic acid	1.00	0.62	0.84
glycolic acid	1.00	1.13	1.01
D-pipecoli acid	1.00	0.69	0.00
hydracrylic acid	1.00	6.98	1.05
butenoic acid	1.00	2.46	9.71
2-pyrrolidinone-5-carboxylic acid	1.00	1.18	0.43
others			
methyl-amino acetate	1.000	0.013	0.218
acetamide	1.000	0.577	18.533
triazol-3-amine	1.000	0.910	2.026
maltol	1.000	1.291	1.125
2-mercaptophenol	1.000	1.108	1.276
urea	1.000	very low	3.444

Supplementary Table 1. Metabolite content in extracts of *P. vulgaris*, *C. cajan* and *V. unguiculata*, analysed by GC/MS. Relative amounts of each metabolite are referred to *Phaseolus vulgaris* content, which was set to 1.

Analytes	Target ion (m/z)
glycine	246
L-leucine	200
L-threonine	404
L-alanine	232
L-proline	184
L-asparagine	417
L-aspartic acid	418
hypoxanthine	307
D-pyroglutamic acid	300
methylthiouracil	313
Citric acid	459
Malic acid	419
DL-glyceraldehyde	115
Lactic acid	261
glycerol	377
Linolealidic acid	337
Linolenic acid	335
Stearic acid	341
Palmitic acid	313
D-pipecolic acid	186
2-pentendioic acid	315
2-thiobarbituric acid	429
L-dihydroorotic acid	443
Glycolic acid	247
2-pipecoli acid	186
Hydroacrylic acid	261
Butenoic acid	289

2-butenoic acid	273
2-pyrrolidinone-5-carboxylic acid	186
Methyl-amino-acetate	146
acetamide	116
Triazol-3-amine	213
maltol	183
2-mercaptophenol	297
urea	231

Supplementary Table 2. Identified analytes by GC/MS to measure the peak area

Strain	Genotype	Source
wt	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	Euroscarf
snf1Δ	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ01 snf1::HPH	This study
atg1Δ	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ01 atg1::KanMX	This study
ras2Δ	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ01 ras2::KanMX	This study
tor2Δ	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ01 tor2::KanMX	This study
wt [empty]	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242]	This study
wt [αSyn]	BY4742 MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 [pYX242-SNCA]	This study

Supplementary Table 3. Yeast strains used in this study.

Gene	5'-Forward-3'	5'-Reverse-3'
Sirt1	CATTATGCCGCATTTCGCCA	GAAGGTGTTCACTGAGGCCA
Foxo	AGGCTGACCCACACAGATAAC	GGCTCCACAAAGTTTTCGGG
Notch	CGCTTCCTGCACAAGTGTC	GCGCAGTAGGTTTTGCCATT
HO	ATGTCAGCGAGCGAAGAAACA	TGGCTTTACGCAACTCCTTTG
Trxr	TGGATCTGCGCGACAAGAAAG	GAAGGTCTGGGCGGTGATTG
RPL32	GCCCACCGGATTCAAGAAGT	CTTGCGCTTCTTGGAGGAGA

Supplementary Table 4. List of primers for real-time PCR.

5. Lorenzo Guzzetti, Davide Panzeri, Marynka Ulaszewska, Grazia Sacco, Matilde Forcella, Paola Fusi, Nicola Tommasi, Andrea Fiorini, Luca Campone, Massimo Labra. Assessing dietary bioactive phenolic compounds and agricultural sustainability of an African leafy vegetable: the case of *Corcorhus olitorius* L.

In this work, the effect of agronomic growth conditions is assessed on the amount of produced biomass and more deeply on the antioxidant composition of *C. olitorius* leaves. Moreover, the phenolic fractions obtained from *C. olitorius* leaves are tested on human colon cell lines to assess bioactive properties both at the viability and antioxidant metabolism level.

General considerations: Also *C. olitorius* shows to be suitable for conservation agriculture strategies. Interestingly, the antioxidant composition of its leaves is not affected by agronomic treatments, probably meaning that these conditions are not stressful to the plant. The nutraceutical properties of antioxidant fractions of leaves suggest that this species is suitable for the implementation in nutritional prevention programs.

Assessing dietary bioactive phenolic compounds and agricultural sustainability of an African leafy vegetable: the case of *Corchorus olitorius* L. [SUBMITTED TO THE JOURNAL OF THE SCIENCE OF FOOD AND AGRICULTURE]

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Abstract

BACKGROUND: *Corchorus olitorius* L. is an African leafy vegetable of nutritional interest. To assess its agricultural suitability to sustainable cultivation conditions, its total biomass and phytochemicals content in response to conservation agriculture practices (i.e., no tillage and cover crop maintenance) and low water regime were evaluated and compared with response under conventional agriculture management.

RESULTS: Hydric stress and no-tillage did not affect neither plant growth nor antioxidant metabolites content compared to conventional agricultural practices. In both conditions, leaves were found to be a great source of phenolic compounds. The effect of these phenolic fractions was assessed on two colon cell phenotypes to evaluate putative nutraceutical properties. Polyphenols-enriched extracts (PEE) displayed selective cytotoxic activities against tumor Caco-2 cells but not on the healthy CCD841 line. PEE were able to trigger oxidative stress and to inhibit the activity of glutathione-independent antioxidant enzymes on Caco-2 cells.

CONCLUSIONS: This work highlights the suitability of *C. olitorius* to conservation agriculture practices and extends the nutraceutical value of this species, already reported by previous studies, to human colon cell systems.

Key Words: *Corchorus olitorius,* conservation agriculture, tillage, water stress, phenolic compounds, bioactives.

Introduction

In Anthropocene, providing a healthy diet from sustainable food systems is a global challenge, also mentioned in the UN Sustainable Development Goals (i.e., SDG2). As a matter of fact, hunger is still on the rise and many people consume low-quality diets causing micronutrient deficiencies [1]. Also in developed countries, population is inadequately nourished and many environmental systems and processes are pushed beyond safe boundaries by food production. This leads to deforestation, pollution, alteration of ecosystems and increases the risks to human health. It is known that pandemic risks such as SARS and COVID-19 have derived from the enhancement of the interactions between wild or domestic animals and humans in response to the growing demand for new areas of cultivation and breeding, as well as the search for new food resources [2,3]. In this context, the scientific targets for healthy diets and sustainable food systems are integrated into a common framework of 'one health'; therefore the innovation of the agri-food system in the modern society allows to merge the issue of malnutrition with the sustainability one. Moreover, diet is not only aimed at guaranteeing a suitable source of macronutrients but also at ensuring a balanced micro-nutrients intake [4], including bioactive molecules able to promote human health and solve global nutritional issues, such as hidden hunger, stunting and famines. Not only vitamins and minerals, but also dietary nutraceuticals such as plant antioxidant, anti-inflammatory and anti-aging compounds have been proved to contrast the outbreak of Non-Communicable Diseases (NCDs), as well as metabolic disorders (e.g., obesity), so that their uptake is recommended by institutions such as the World Health Organization (WHO) [5-8].

In order to promote a healthy diet and food security issues, researchers are trying to identify new food items rich in bioactive molecules as well as more sustainable in terms of food production. Local biodiversity and agrobiodiversity represent an important source of these secondary compounds [9] distributed in different plant organs (i.e. leaves) and not only in the common edible portions such as fruits and seeds. An opportunity to increase the uptake of bioactive compounds in the diet comes from those regions not interested by the Green Revolution, or at least where it did not impact consistently in terms of biodiversity erosion, as it happened in many developing countries, which experienced a lag in the benefits provided by the modern crop varieties of the Green Revolution due to political and pedoclimatic conditions [10]. Therefore, there are many autochthonous edible plants that are unexplored in the context of agrobiodiversity and that could contribute to improve agricultural issues worldwide [9].

In this work, we evaluated the healthy properties of *Corchorus olitorius* L., an indigenous African leafy vegetable known as jute mallow. This plant is largely diffused in the middle East and in African countries [11-13] and is mainly cultivated in smallholding farms for self-consumption. It is made into a common mucilaginous soup or sauce in some West African cooking traditions [14]. It has a high content of fiber, vitamins and antioxidant compounds [15-16], is highly resistant against environmental stressors such as drought and water deficiency [4] and requires low fertilization to grow [17].

The aim of this study was to deepen the metabolic profile of jute mallow phenolic fractions obtained from leaves, following the traditional food preparation procedures (i.e. boiling) adopted in African countries where this plant is mostly consumed [18,19]. At the same level, we tested the agronomic sustainability of jute

mallow through the analysis of plant responses to conservation agriculture practices (i.e. no tillage plus cover crops) and water scarcity conditions in the Mediterranean context with the aim of identifying the most suitable and sustainable agronomic conditions to enhance agronomic yield and leafy bioactive compounds content.

Finally, we evaluated the bioactivity of jute mallow leaves on human cells viability and, specifically, we investigated the response of human colon cell lines to the leafy phenolic extract to assess whether its consumption can be related to a reduced risk of digestive trait malignancies, as already shown for other vegetables [20]. This hypothesis derives from a previous study of [21], which highlighted a selective cytotoxic activity of jute mallow leaves against cancer liver cell lines (HepG2), without displaying undesirable effects on the healthy one FL83B. The reduction in the tumor cell line viability was found to be associated with the activation of mitochondria dependent apoptosis pathway leading to the activation of procaspase 3 and 9 and to the subsequent DNA fragmentation. However, no information has been reported about the effect on gut cancer despite being the first site where foods act after gastric digestion. Therefore, our evaluation allows to provide more precise information on the effects of such green leafy items, in preventing disease and enhancing human well-being.

2. Material and methods

2.1. Plant Material and growing conditions

Seeds of *C. olitorius* (accession ID. SUD-2) were obtained from the seed bank of the Word Vegetation Center (AVRDC; Arusha, Tanzania). These were used to perform agronomic and phytochemical analysis. Plants were cultivated with different agricultural approach (conventional vs conservation agriculture practices) following the same experimental design described in [22]. Briefly, between May and August 2018, the trial took place in a long-term experimental field in San Bonico, Piacenza, Italy (45◦00018.0" N, 9◦42012.7" E; 68 m above sea level). A total of 8 plots (15m x 3m), were set up. Four of these were under conservation agriculture management (i.e., 8-yr no tillage plus cover crops - hereafter NT and cover crop maintenance) while the other four were under conventional agriculture management (i.e., conventional tillage - hereafter CT and absence of cover crops). Moreover, to test the ability of jute mallow to resist against water stress, two separated subplots for each growing condition were defined: one subplot was normally watered (hereafter W), while the other was not watered to mimic stressful conditions (hereafter NW). Soon after before flowering, plants leaves were manually collected and stored at -20°C before analyses.

In order to evaluate the effect of the growth conditions on plant yield, the total biomass obtained by *C. olitorius* cultivation was measured by harvesting three randomly selected 2m × 1m squares from each subplot. Total above-ground biomass was manually cut at the soil surface and weighted. Then, the dry biomass

was gravimetrically determined by drying a representative subsample of biomass at 65 °C, until constant weight.

2.2. Phytoextraction

Pooled leaves mixtures obtained for each subplot were processed three times independently. In particular, leaves were washed and boiled in water at 100°C for 15 min in order to mimic African traditional culinary habits [14,18]. Leaves were dried in an oven at 50°C overnight and then grinded and stored at -20°C until analysis. Each pool of leaves was extracted according to the protocol of [23]. Briefly, 1 gram of powdered leaves was suspended in 4.8 mL of methanol 67% and 4.8mL of chloroform. The powder was extracted on an orbital shaker (VDRL 711+, Asal, Italy) for 15 minutes at constant rotation (400 rpm).. Samples were extracted for a second time by adding further 2.4 mL of the above mentioned extraction solution. The alcoholic portion of the extract was evaporated by using a rotary evaporator (40°C, 120 rpm, 1000 bar), then the remaining aqueous fraction was freeze dried. Finally, samples were resuspended in water in order to obtain a concentration of extract equal to 6.7 mg/mL. All chemicals were purchased from Sigma-Aldrich Merck, Germany.

2.3. Total phenol content evaluation

The total phenol content of each extract was estimated through the Folin-Ciocalteau assay as described in [24]. Briefly, each sample was diluted to a concentration equal to 0.2 mg/mL and a calibration curve was made up by using

increasing concentration of gallic acid (from 0 to 100 µg/mL). The analysis was performed by adding in a quartz cell 400 µL of ultrapure milli-Q water, 80 µL of sample or standard, 40 µL of Folin-Ciocalteau reagent and 480 µL of a solution 10.75% Na_2CO_3. After an incubation of 30 minutes at room temperature, samples were read against the blank at a wavelength of 760 nm. Results are expressed as g GAE (Gallic Acid Equivalent) per g of leaves or in 100 g of extract. All chemicals were purchased from Sigma-Aldrich Merck, Germany.

2.4. DPPH Radical Scavenging Activity

The Radical Scavenging Activity of jute mallow total extracts was evaluated through the DPPH assay. Briefly, 50 µL of samples at a concentration of 0.2 mg/mL were added to 950 µL of DPPH (2,2-diphenyl-1-picrylhydrazyl) 0.1 mmol/L.
A calibration curve was made up by using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard reference in a range of concentrations between 0 and 500 µmol/L. After an incubation of 30 minutes at room temperature, samples were read against the blank at a wavelength of 515 nm. Results are expressed as g TE (Trolox Equivalent) per g of leaves. All chemicals were purchased from Sigma-Aldrich Merck, Germany.

2.5. Solid Phase extraction and UHPLC UV-DAD analysis

In order to enrich the polyphenolic fraction of jute mallow extracts, a SPE-based purification procedure was performed. SPE cartridges (Strata-X, Phenomenex, USA)

were conditioned with 3 mL methanol and equilibrated with 3 mL of ultrapure milli-Q water. Samples (up to 200 mg of extracts per time) were loaded and then the cartridge was washed with 3 mL water and finally eluted with 4.5 mL methanol pH 2. The collected fraction was evaporated up to dryness in a speed-vac centrifuge (Eppendorf, Germany). Dried samples were resuspended to reach a concentration of 0.3 mg/mL for HPLC-DAD MS analysis and filtered through a 0.22 µm filter before UHPLC anaysis. Their total phenol content (see Paragraph 3) was evaluated prior to being administered to cells. The eight most concentrated Polyphenols Enriched Extracts (PEE) of the sixteen plots were evaluated for their bioactive effects on healthy and colorectal cancer cell lines. PEE were subjected to the analyses of the main phenolic compounds through a UHPLC-UV analysis

2.6. Qualitative and quantitative analysis of phytoextract

Main phenolic compounds of PEE were characterized using a LTQ OrbiTrap XL mass spectrometer (ThermoFisher Scientific, Milan, Italy) connected to an UHPLC system 1260 Infinity LC System (Agilent, USA) consisting of a high pressure pump, an autosampler, a diode array detector and a column oven compartment. Chromatographic separation was performed through a Kinetex Biphenyl 100 A (50x2.1mm, 2.6 µm, Phenomenex). Mobile phases consisted of water 0.1% (A) and methanol 0.1% (B) both with 0.1% v/v formic acid. After injection (5 µL) the analytes were eluted using the following gradient 0-2 min 5% B, 2-5 min linear increase to 10% B, 5-7 min 10% B, 7-10 linear increase to 25% B, 10-12 min 25% B 12-15 linear increase to 30% B, 15-20 30% B, 20-25 linear increase to 40%, 25-30 min increase up to 95% B. At the end of each run the column was washed for 4 min with 95% B

and re-equilibrated for 5 min. The flow rate was kept at 0.3 mL/min and the column oven was set at 30°C for all chromatographic runs. UV spectra were acquired in the range between 200 and 600 nm and two wavelengths (280 and 330 nm) were employed for detection and quantification of target analytes. High resolution mass spectrometry, equipped with ESI source, operating both in positive and negative ion modes, with spectra acquired in a mass range between 80-800 Da at a resolution of 30000 FWHM and data dependent scan with 7500 FWHM resolution, was used to for the chemical characterization of the main phenolic compounds in PEE. The optimized value of ESI source were the following: source voltage 3.5 kV, capillary voltage 42 V, tube lens voltage 45.6V, capillary temperature 280 °C, sheath and auxiliary gas flow (N2) 30 and 10 respectively (arbitrary units). Product ions in DDA scans were generated in the LTQ trap at collision energy 35 eV using an isolation width of 1 Da. Detected compounds were tentatively identified along their retention time (Rt), accurate mass (positive and negative ionization), calculated molecular formula, error ppm (between detected mass and calculated accurate mass), MS/MS fragmentation and bibliographic references. Furthermore, phenolic compounds identified by mass spectrometry analysis were confirmed by reference standards. The same UHPLC system and conditions were used for quantitative analysis. Calibration external standard method was used to quantify the main phenolic compounds in PEE. A mixture of 8 standards (chlorogenic acid (CA), neochlorogenic acid (NCA), cryptochlorogenic acid (CCA), quercetin-3-O-galactoside (Q3Gal), isoquercitrin (Q3Gly), quercetin-3-O-malonyl-glycoside (Q3MGly), 3,5-dicaffeoylquinic acid (3,5-DCQA), and kaempferol-3-O-glycoside (K3Gly) were used to produce a calibration curve in a concentration range between 1 and 100 µg/mL with 6 calibration point. The amount of quantified compound in

samples was expressed as µg/mL ± SD. The statistical analysis of variance (ANOVA) was performed to determine the goodness-of-fit and linearity of the curves.

2.7. Bioactivity analysis

The effect of each extract on colon cells was evaluated by using CCD841 (ATCC® CRL-1790™) human healthy intestinal mucosa cell line and Caco-2 (ATCC® HTB-37™) human colorectal cancer cell line. These were grown in EMEM medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin. All cell lines were maintained at 37°C in a humidified 5% CO_2 incubator. ATCC cell lines were validated by short tandem repeat profiles that are generated by simultaneous amplification of multiple short tandem repeat loci and amelogenin (for gender identification). All the reagents for cell cultures were supplied by Lonza (Lonza Group, Basel, Switzerland).

2.7.1. Cell viability assay

Cell viability assay was investigated using MTT-based *in vitro* toxicology assay kit (Sigma, St. Louis, MO, USA), according to manufacturer's protocols.

The different cell lines were seeded in 96-well microtiter plates at a density of 1 x 10^4 cells/well, cultured in complete medium and treated after 24 h with 100 µg/mL of PEE. After 48 h at 37°C, the medium was replaced with 100 µL of complete medium without phenol red containing 10 µL of 5 mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide). After 4 h incubation more

for CCD841 and 2 h for CRC cell line, formazan crystals were solubilized with 10 % Triton X-100, 0.1 N HCl in isopropanol and absorbance was measured at 570 nm using a microplate reader. Cell viabilities were expressed as a percentage against untreated cell lines used as controls.

2.7.2. Oxidative stress assay

The generation of intracellular reactive oxygen species (ROS) was detected by the oxidation of 2',7'-dichlorofluorescin diacetate (H2DCFDA) (Sigma Chemical Co., St. Louis, MO), an indicator for both reactive oxygen species and nitric oxide (•NO). Caco-2 cell line was seeded in 96-well black microtiter plates at a density of 1 x 10^4 cells/well, cultured in complete medium and incubated after 24 h with 5 µM H2DCFDA in PBS for 30 min in the dark at 37°C. After two washes in PBS, cells were treated 100 µg/mL of extract for 2 h or 1 mM H_2O_2 for 1 h for positive control. The fluorescence (λem = 485 nm/ λex = 535 nm) was measured at 37 °C using a fluorescence microtiter plate reader (VICTOR X3) and analyzed by the PerkinElmer 2030 Manager software for Windows.

2.7.3. Antioxidant enzymes assays

To evaluate the effect of PEE on enzymatic activities, Caco-2 cell line was seeded at 1×10^6 cells/100 mm dish and treated for 48 h with the extract at 100 µg/mL. The cells were rinsed with ice-cold PBS and lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% Glycerol, 1% NP-40, containing protease inhibitors (1 µM leupeptin, 2 µg/mL aprotinin, 1 µg/mL pepstatin and 1 mM PMSF). Homogenates were obtained by passing 5 times through a blunt 20-gauge needle fitted to a

syringe and then centrifuged at 15,000g for 30 min at 4°C. Supernatants were used to measure enzymatic activities: glutathione S-transferase (GST) were assayed as previous described [25]; glutathione reductase (GR) was assayed according to [26]; glutathione peroxidase (GPox) as reported in [27]; superoxide dismutase (SOD) as previously described [28]; catalase (CAT) was according to [29]. Enzymatic activities were expressed in international units and referred to protein concentration as determined by the Bradford method [30]. All assays were performed in triplicate at 25 °C in a Jasco V-550 Spectrophotometer and analyzed by the Spectra Manager (version 1.33.02) software for Windows.

2.8. Statistical analyses

All the data were analyzed by using R version 3.3.3. Total aerial biomass yield as a function of the different agronomic treatment was estimated through a Linear Mixed Effect Model (LME) considering the plot as random effect and the growth treatment as the fixed one. Response variable was assumed normally distributed. Data from Folin-Ciocalteau and DPPH assay were analyzed by a Generalized Linear Mixed Effect Model (GLMM) assuming a binomial distribution of the response variable. Switch to beta-binomial distribution was provided to avoid overdispersion. The random effect was the plot in which plants were grown and the fixed effect was the growth treatment. Data from the MTT assay were analyzed again by a GLMM considering cells viability as response variable, while the independent variable was the cell line. The random effect was again the plot. Then each single plot was analyzed by a Generalized Linear Model (GLM) assuming a binomial distribution of the response variable (% cells viability). The fixed effect was the cell line. Switch to quasi-binomial distribution was provided to avoid

overdispersion. *p*-values were adjusted considering the Bonferroni's correction for multiple comparisons. Packages used were TMB, glmmTMB, ggplot2. Data about enzymatic activity assessment were analyzed by using the fold parameter (activity of the enzyme from treated cells normalized against the activity of the control). To evaluate the existence of differences among the experimental groups and the control one-way ANOVA and Dunnett's post hoc test were used. The same statistical analysis was performed to evaluate the effect of extract treatments on the level of ROS produced by cells compared to the untreated control. Data were analyzed on R by using the multcomp package.

3. Results

3.1. Agronomic performance and antioxidants composition of *C. olitorius* grown under different agronomic conditions

Figure 1A shows the results obtained by estimating the aerial biomass of jute mallow plants grown as a function of the two growth conditions (conventional tillage- CT and no tillage - NT) with and without watering (W and NW), respectively. No significant differences were detected among treatments in terms of total biomass (χ^2=4.24, p = 0.37).

Concerning bioactive compounds, data suggested that the total phenol content of *C. olitorius* boiled leaves is around 0.25% of the total leaves weight. No significant differences were detected among cultivation treatments (χ^2 = 0.38, p = 0.944). The

radical scavenging activity follows the same trend identified for the total phenol content and in particular is comparable among treatments with values ranging between 2.5 and 3.2 mg TE (Equivalent of Trolox, a chemical analogue to tocopherol) per g of leaves. Also in this case, none of the treatment was found to significantly affect the radical scavenging activity of the extracts (χ^2 = 0.3, *p*= 0.9599; see Figure 1C).

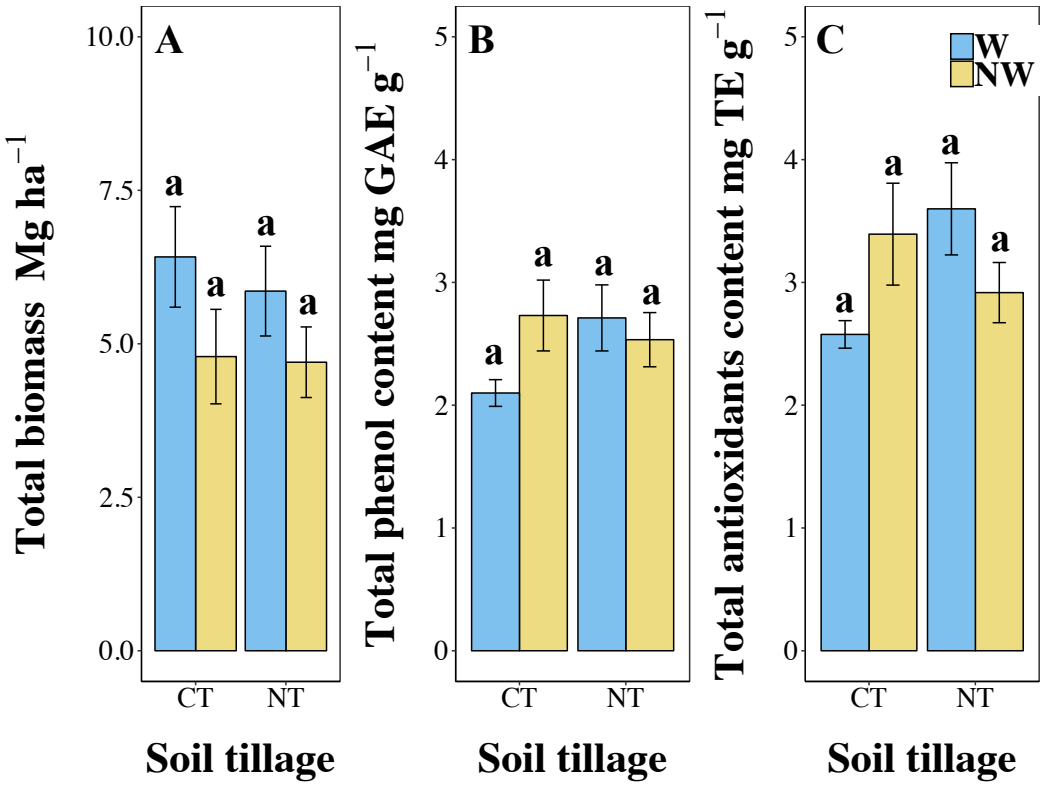

Figure 1: A) evaluation of the total aerial biomass (Mg ha^{-1}), **B)** total phenol content (expressed as Gallic Acid Equivalents per gram of dry leaves), **C)** total antioxidants content (expressed as Trolox Equivalents per gram of dry leaves) of jute mallow plants grown under the four different agronomic treatments. Data are expressed as the mean ± SEM. CT = conventionally tilled; NT = no tilled; W = watered; NW = not watered.

3.2 Qualitative and quantitative profile of jute mallow extract by UHPLC -DAD-$HRMS^n$

In order to estimate the chemical composition of main compounds occurring in leafy extracts and to identify the compounds responsible for the abovementioned antioxidant activity (see Figure 1C), a HRMS untarget screening was performed on the extract from each experimental growth condition**.** The leafy extracts were analyzed by UHPLC-DAD-$HRMS^n$ to investigate their qualitative phenolic profile. (Table S1). Metabolite assignments were made by comparing retention time, UV/Vis spectra and MS data (accurate mass and MS^n fragment ions) of the compounds detected with standard compounds, whenever available, and jute mallow compounds reported in the literature and databases. The most abundant phenolic with main information (name, retention times, UV and MS data) for individual components are listed in Table S1. Main compounds were quercetin and kaempferol-(malonyl-)hexosides, chlorogenic acids, dicaffeoylquinic acids and feruloyl-quinic acids isomers. Results of HRMS untarget analysis did not reveal statistically significant differences between agricultural treatments. Afterwards,

the chemical characterization of main compounds occurring in PEE was carried out by UHPLC-UV analysis. Quantitative data are reported in Table 1, whereas Figure S1 shows the phenolic profile of the main compounds both in standard solution and PEE at 280 (A) and 330 nm (B). A total of 11-20 peaks per sample were detected and 8 of these were identified. PEE showed a total phenolic content calculated as the sum of individual phenolic compounds ranging between 74 and 99 μg/mL.

PEE showed a total phenolic content calculated as the sum of individual phenolic compounds ranging between 74-99 μg/mL. In particular, Q-3-O-MG was the most abundant phenolic compound in all the analyzed samples (32-39 μg/mL). Conversely, the content of chlorogenic acids derivatives in all the treatments was very low, ranging between 0.18 and 0.65 μg/mL as well as the level of K-3-O-Gly (1.9-3.3 μg/mL). However, results of quantitative analysis did not show statistically significant differences among the investigated agronomic treatments.

Compounds (μg/mL)	**NTW**	**CTW**	**CTNW**	**NTNW**
neochlorogenic acid	0,296 ± 0,296	0,345 ± 0,345	0,188 ± 0,188	0
cryptochlorogenic acid	0,646 ± 0,405	0,563 ± 0,37	0,242 ± 0,242	0
chlorogenic acid	1,161 ± 0,608	0,66 ± 0,214	0,577 ± 0,239	0,18 ± 0,18
3,5-DCQA	3,041 ± 0,779	2,612 ± 1,554	2,171 ± 0,026	2,101 ±0,278
quercetin-3-O-galactoside	24,721 ± 2,733	25 ± 8,481	29,547 ± 4,753	20,35 ± 1,68
isoquercitrin	22,542 ± 4,842	28,35 ± 2,0735	24,9425 ± 2,668	16,59 ± 3,772
quercetin-3-O-malonyl-glycoside	32,142 ± 7,405	33,13 ± 8,97	39,496 ± 7,884	33,562 ± 1,046

astragalin	2,662 ± 0,646	1,632 ± 1,395	3,315 ± 0,229	1,988 ± 0,464
TOTAL	87,211	92,29	99,478	74,771

Table 1. Concentration (µg/mL) of the main phenolic species occurring within PEE in 100 µg/mL GAE. Data represent the concentration of each compound which cells were treated with and are expressed as the mean ± SEM. NTW = no tilled and watered plots, CTW = conventionally tilled and watered plots, NTNW = no tilled and not watered plots, CTNW = conventionally tilled and not watered plots.

3.3. MTT viability assay

PEE from the different experimental conditions were evaluated for their bioactive properties on CCD841, a healthy colon cell line used as reference, and Caco-2, a colorectal cancer cell line.

MTT assay performed on cells treated with PEE showed significant effects ($p < 0.001$) on Caco-2 cells at a concentration of 100 µg/mL with an average viability reduction equal to 39%, compared to the healthy cell line, which maintained a percentage of viability higher than 80%, therefore it was not negatively affected by the treatment with *C. olitorius* PEE.

The analysis performed on single plots (Figure 2) highlighted a stronger effect on Caco-2 from plants grown under no tillage management (NT). Three of the four plots analyzed were able to cause a viability reduction on the Caco-2 cell line

significantly higher than the healthy one. Only 1 plot from conventional agriculture management (CT) was found to be significantly more cytotoxic against Caco-2 cells compared to the healthy control. No significant effects were found to be driven by drought treatment.

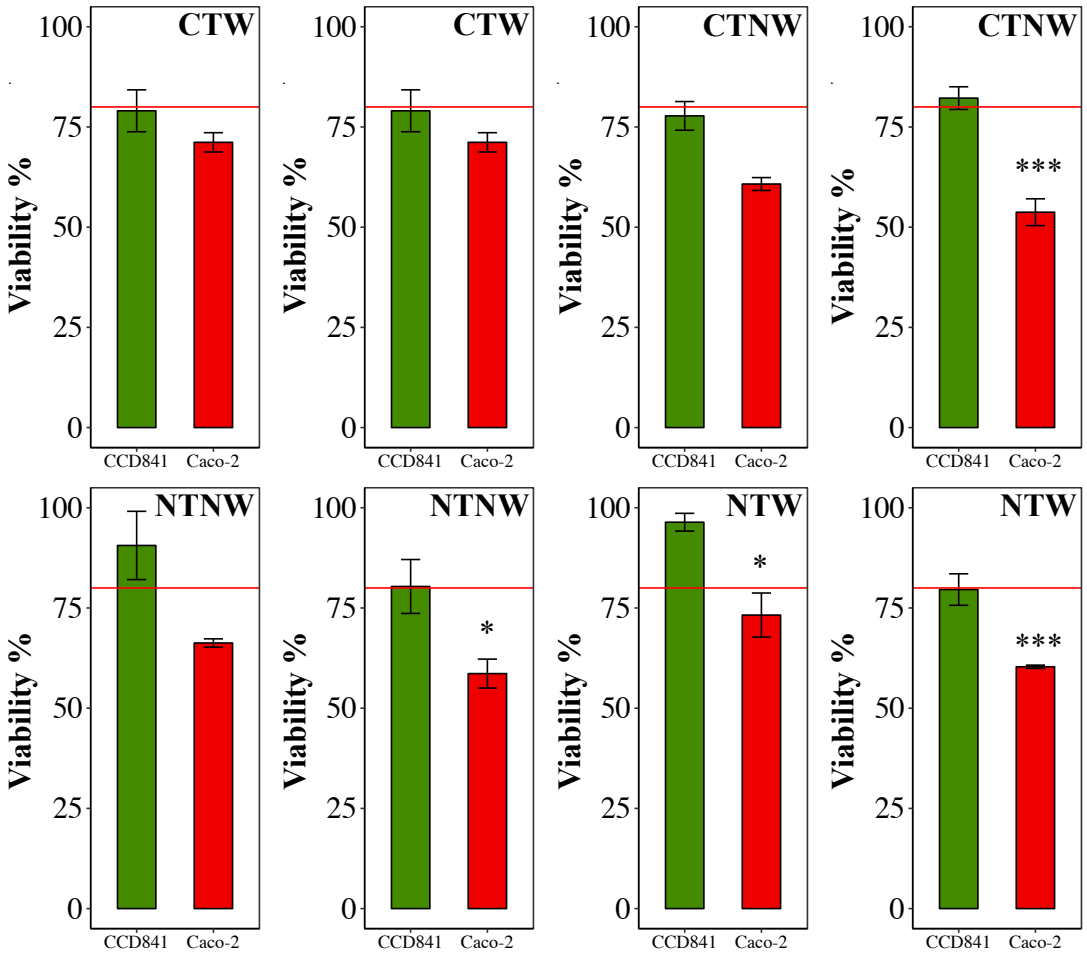

Figure 2: MTT viability assay on two human colorectal cell lines: CCD841 (green, healthy line), Caco-2 (red, colorectal cancer cell line). All cell lines were treated with 100 µg/ml GAE in the medium. Data are mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** p

< 0.001. CTW = conventionally tilled and watered plots, CTNW = conventionally tilled and not watered plots, NTNW = no tilled and not watered plots, NTW = no tilled and watered plots.

3.4. Oxidative stress analysis

An oxidative stress analysis was performed to better clarify the action mechanism of jute mallow PEE on cancer cells. The fluorescence analyses performed on Caco-2 cells revealed a significant increase in the production of Reactive Oxygen Species (ROS) compared to the control ($F = 38.53$, $p < 0.001$) after 2 hours treatment. Each tested sample induced the same effect with quantitative differences expressed in Figure 3A.

The intracellular detoxification systems were also evaluated to clarify the cellular response after PEE treatments in the Caco-2 cell line.

SOD activity was found to be significantly affected by PEE treatments ($F = 11.54$, $p < 0.001$) (Figure 4B). In particular, SOD activity was negatively affected by treatment with PEE from tilled plots, both watered ($p = 0.01$) and not watered ($p = 0.023$) compared to the control, while no significant effects were elicited by the treatment with PEE from no-tilled plots, both not watered ($p = 0.562$) and not watered ($p = 0.127$).

CAT was found to be the most affected enzyme ($F = 13.13$, $p < 0.001$) (Figure 3C). Almost all the conditions were able to display a significant reduction on its enzymatic activity, more marked from PEE coming from the watered conditions, irrespective of the tillage ($p < 0.001$) or no-tillage ($p < 0.001$) treatment. PEE from not-watered plots displayed a weaker effect, significant for those obtained from

no-tilled condition (p =0.04) and only marginally significant from those deriving from tilled condition (p = 0.058).

GST activity was found to be slightly affected in a positive manner by PEE treatment (Figure 3D). However, the only experimental group showing a significant difference (t = 2.833, p = 0.037) compared to the untreated control was TW which was the PEE group showing the least ability to discriminate with the healthy cell line (Figure 3). Conversely, the other groups showed a not significant difference in the GST activation compared to the control. Furthermore, despite some fluctuations among treatments, GR activity did not show any significant variation compared to the control in none of the treated samples (F = 0.67, p = 0.622) (Figure 3E). Therefore, GR did not appear to be a candidate to explain the decrease in viability of Caco-2 cells treated with PEE. Also, GPox activity was found not to be influenced by extracts obtained from plants deriving from the different growth treatments (F = 1.331, p= 0.304) (Figure 3F). Overall, enzymes responsible for glutathione metabolism did not display significant variations under PEE treatment compared to the control.

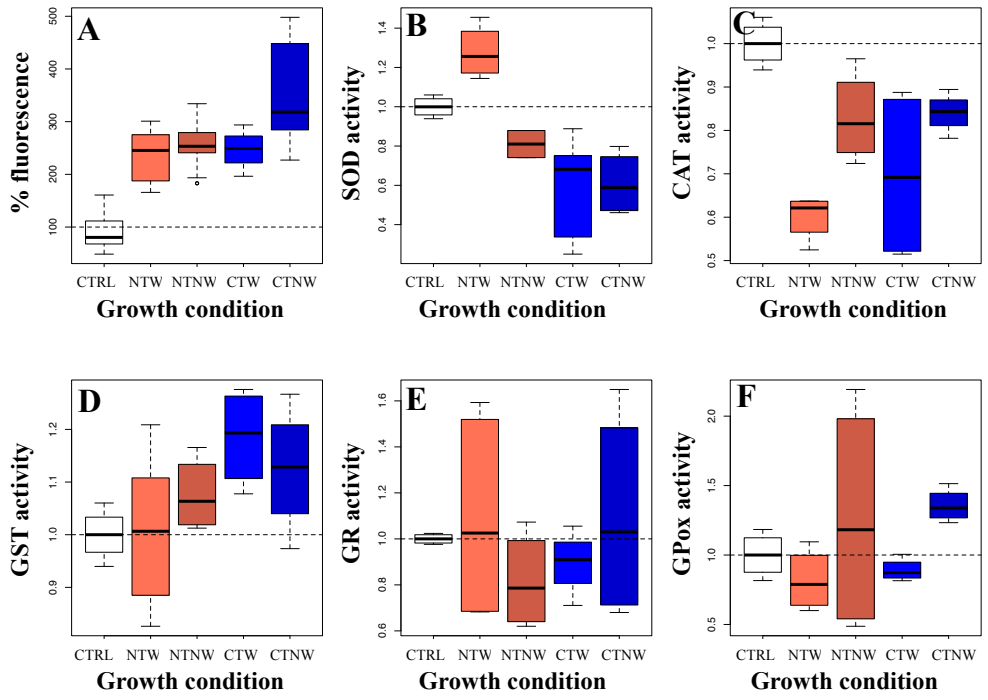

Figure 3: Oxidative stress detection (A) and enzymatic activity assays expressed as fold units compared to a non-treated control (CTRL) on five antioxidant cellular enzymes (B: superoxide dismutase, C: catalase, D: glutathione S-transferase, E: glutathione reductase, F: glutathione peroxidase) performed on Caco-2 cells treated with 100 µg/mL GAE in the culture medium. CTW = conventionally tilled and watered plots, CTNW = conventionally tilled and not watered plots, NTNW = no tilled and not watered plots, NTW = no tilled and watered plots.

4. Discussion

4.1 Responses of *C. olitorius* to conservation agriculture management

In this work, we highlighted that *C. olitorius* cultivation may be performed with conservation agriculture practices (without reducing total biomass production) and in harsh conditions resembling those of arid and semiarid regions, where not all the leafy greens are able to grow well [31]. According to [32], conservation agriculture is the one of the most promising management strategies of worldwide agroecosystems, since it allows to improve and sustain productivity, to increase profits and food security while preserving and enhancing environmental resources [33-35]. In terms of agriculture sustainability in Mediterranean countries, no-tillage is reported to enhance water soil content in response to climate change [22] and to preserve soil from erosion and leaching [36]. In our work, we highlighted that *C. olitorius* is rich in several polyphenols and that their amount and composition are not modified by stressful cultivation conditions, such as no-tillage and the reduction of water irrigation. It is important to underline that the *C. olitorius* genotype used in this study was selected from the seed bank of the Word Vegetation Center in Arusha (Tanzania) to tolerate water stress and therefore the metabolic response to water scarcity was not particularly evident. However, studies conducted on other *C. olitorius* cultivars showed an increase in total polyphenols and flavonoids content as a response to water scarcity conditions [11]. A dedicated screening of these cultivars for their suitability to be grown in the Mediterranean context could also be performed to select the most promising accession in terms of valuable secondary metabolites content.

Concerning the nutritional properties, the main critical element regarding jute mallow is represented by the consumption habits that involve boiling the leaves. In the case of *C. olitorius*, recent data showed that the total polyphenols content of fresh leaves ranges from 5.41 to 7.78 mg GAE/g DW depending on genotype characteristics [37]. Considering these reference values, our data suggests that boiling reduced these values by at least 55-65% the amount of polyphenols. This is in agreement with a similar research conducted on other leafy vegetables [38]. Currently, there are no indications of *C. olitorius* leaves consumption alternative to boiling. However, innovative technologies could be applied to identify methods of preparation and consumption able to preserve antioxidant metabolites, such as microwave cooking processes which were found to be suitable methods to preserve (or also to ameliorate) the amount of phenolic compounds and the antioxidant capacity of several green leafy vegetables [39].

4.2. Healthy properties of *C. olitorius*

Carcinogenesis consists of many phases (initiation, promotion, and progression), each one associated with specific prevention and intervention strategies. Chemoprevention involves the use of specific natural products or synthetic chemical agents to reverse, suppress, or prevent premalignancy before the development of invasive cancer [40,41]. In this study, *C. olitorius* leafy phenolic fractions were found to induce a significant reduction in the viability of Caco-2 cancer cells without any detrimental effect on the healthy cell line. These properties have been observed despite the preliminary boiling and make *C. olitorius* a promising candidate species in the field of nutritional prevention. A similar effect

in terms of selectivity in the reduction of HepG2 liver cancer cell line viability was shown in a previous study performed by [21] which found that the mechanism of action promoted by jute mallow extracts is the mitochondria-dependent apoptosis pathway. In our study, the selective cytotoxic activity triggered by *C. olitorius* PEE appeared to be mediated by a sudden increase in the ROS level, still high after 2 hours treatment and through a successive decrease in the activity of glutathione-independent antioxidant enzymes. This activity may appear as a paradox because phenolic compounds are known to be among the best antioxidant plant phytochemicals. However, it is important to note that the tumor environment shows different redox status compared to healthy tissues and this study confirms previous observations highlighting that - despite their well-known antioxidant properties on healthy tissues - these secondary compounds are able to induce pro-oxidant responses on tumor cells, triggering programmed cell death mechanisms such as apoptosis [42,43].

Concerning the bioactive compounds, many flavonoids were identified within PEE, mostly belonging to the family of quercetin derivatives. We underline that Q-3-O-Gal, for instance, accounted for 20-30% of the total phenolic composition. It is likely that this compound (together with the other quercetin derivatives) may be responsible for the selective cytotoxicity against the Caco-2 cell line with no detrimental effects on the healthy one. The anti-inflammatory and antioxidant properties of this compound extracted from jute mallow leaves has been already documented by [44].

Another interesting secondary metabolite identified in PEE is the flavonoid astragalin (K-3-O-Gly), which was found in different amounts in approximately all PEE. This compound has been reported to modulate inflammatory responses by

regulating the expression of NF-κB, iNOS as well as cytokines and chemokines (COX-2, TNF-α, IL-10, and IL-6). Astragalin is also known to be an inhibitor of ERK-1/2 and Akt signaling; therefore, it is a significant compound against cancer proliferation [45]. Finally, CA and its isomers have also been identified in the majority of the analyzed PEE and are probably involved in the selective cytotoxicity exerted by *C. olitorius* phenolic fractions, as previously shown by [46].

The last part of this study is focused at evaluating if cultivation conditions may impact on jute mallow cancer preventive abilities. Our results suggested that samples obtained from conservation agriculture plots are endowed with a higher selectivity (in terms of cytotoxicity) towards the cancer cell line compared to the healthy one. Considering that no significant differences in the polyphenols composition among different field growing conditions were identified, we hypothesize that other unidentified compounds could play a synergic role in reducing the viability of cancer cells by acting on multiple cellular pathways. It is likely that other antioxidant compounds, displaying selective cytotoxic properties against cancer cell lines, have been elicited in response to the growth conditions [47]. The new trends in cancer prevention and therapy is directed at identifying combinations of different phytochemicals, acting simultaneously on several pathways to contrast cancer progression and to overcome drug resistance mechanisms typical of chemotherapy [48,49]. In this regard, deeper differential analyzes will better clarify which compounds vary between the tested growth conditions leading to differential bioactive responses.

Overall, *C. olitorius* fully meets the interests of modern food sciences in terms of sustainability and health. Furthermore, this plant constitutes an important resource not only for developing countries, but also for urbanized areas, where it could

improve diets nutritional quality and display preventive effects against citizens emerging multifactorial diseases.

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Conflict of interests

The authors declare no competing financial interest.

Supporting Information.

Compound	Rt (min)	Elemental Formula	Ion	*m/z* (Relative Intensity) [a]	Annotation	MS/MS^2 and MS/MS^3 spectra
Quercetin – malonyl-hexose isomers	5.51 5.89 6.35	$C_{24}H_{22}O_{15}$	+	551.1031 (100) *552.1071 (30)* *553.1093 (9)* 303.0490 (25)	$[M+H]^+$ $[M+H-C_9H_{12}O_8]^+$	MS^2 551: 303.0490 (100) MS^3 551-303: 285.0387 (50); 257.0437 (100); 247.0594 (25); 229.0490 (100); 165.0178 (80); 137.0230 (22)
			-	549.0879 (100) *550.0910 (30)* *551.0932 (9)*	$[M-H]^-$	

Chlorogenic acids isomers	3.12 3.71 3.85 4.24	$C_{16}H_{18}O_9$	+	355.1027 (100) *356.1064 (19)* *357.1082 (4)* 163.0387 (30) *164.0421 (3)*	$[M+H]^+$	@Rt 3.12 MS^2 355: 163.0383 (100); 145.0278(15) @Rt 3.71 MS^2 355: 163.0383 (100); 145.0278(8) @Rt 3.85 MS^2 355: 163.0383 (100); 145.0278(15)
			-	353.0870 (100) *354.0901(20)* *355.0921 (4)* 191.0557 (35) *192.0591(3)* 179.0343 (30) *180.0380 (3)*	$[M-H]^-$	@Rt 3.12 MS^2 353: 191.0556(100); 179.0347 (60); 135.0449 (29); 173.0451 (10) @Rt 3.71 MS^2 353: 191.0556(100);

						179.0347 (10); @Rt 3.85 MS^2 353: 173.0451 (100); 191.0556(80); 179.0347 (65); 135.0449 (29); 135.0447 (10) @Rt 4.24 MS^2 353: 191.0556(100); 179.0347 (8); 135.0449 (5);
Quercetin – glycoside isomers	4.99 5.10 5.23	$C_{21}H_{20}O_{12}$	+	465.1033 (100) *466.1067 (24)* *467.1089 (5)* 303.0495 (20)	$[M+H]^+$	MS^2 465: 303.0490 (100) MS^3 465-303: 285.0386 (55); 257.0436 (100);

						247.0594 (30); 229.0490 (100); 165.0179 (60); 137.0230 (20)
			-	n.a.	n.a.	n.a.
Tetrahydroxyisoflavone malonyl-hexose	6.55 6.64 6.79 7.18	$C_{24}H_{22}O_{14}$	+	535.1080 (100) *536.1120 (30)* *537.1144 (6)* 287.0545 (30)	$[M+H]^+$ $[M+H]^-$ $C_9H_{12}O_8]^+$	MS^2 535: 287.0545 (100) MS^3 535-287: 269.0437 (20); 258.0516 (30); 241.0488 (90); 231.0647(30); 213.0540(100); 177.0670(50); 165.01777(100); 153.0179(60); 133.0281(20);

						121.0280(40); 111.0077(15)
			-	533.0931 (100) *534.0965 (30)* *535.0985 (6)*	[M-H]$^-$	MS^2 533: 285.0408 (100)
Dicaffeoylquinic acid isomers	5.60, 5.90, 6.20, 6.50, 7.41	$C_{25}H_{24}O_{12}$	+	*517.1329 (100)* *5181362 (30)* *519.1387 (6)*	[M+H]$^+$	MS^2 517: 499.1217 (100) MS^3 535-499:319.0803 (100); 163.0384(100); 145.0279(10)
			-	515.1181 (100) *516.1213 (30)* *517.1237 (6)*	[M-H]$^-$	MS^2 515: 353.0864(100); 299.0549 (10); 203.0344(10); 173.0449 (15); 191.0533(15); 179.03410

						(10); 255.0657 (15)
Kaempferol hexoside isomers	5.32, 5.70, 6.07	$C_{21}H_{20}O_{11}$	+	449.1080 (100) *450.1115 (25)* *451.1140 (5)*	$[M+H]^+$	MS^2 449: 287.0544 (100) MS^3 449-287: 258.0512 (20); 241.0488 (80); 213.0539(80); 177.0669(75); 165.01777(100); 153.0178(63); 133.0280(20); 121.0280(40); 111.0077(15)
			-	447.0922 (100) *448.0959 (25)*	$[M-H]^-$	MS^2 447: 327.0499 (20); 284.0315(100);

				449.0983 (5)		285.0393(60); 255.0290(13); 227.0341(15); 151.0031(10) MS^3 449-284: 255.0293 (100)
Kaempferol hexoside	6.80	$C_{21}H_{20}O_{11}$	+	449.1080 (100) *450.1115 (25)* *451.1140 (5)*	$[M+H]^+$	MS^2 449: 303.0491 (100); 287.0544 (45)
			-	447.0922 (100) *448.0959 (25)* *449.0983 (5)*	$[M-H]^-$	MS^2 447: 301.0343 (100); 285.0394 (50)
$C_{13}H_{20}O_2$ malonyl hexose conjugate	7.20 7.46	$C_{22}H_{32}O_{10}$	+	457.2070 (100) *458.2108 (26)* *459.2131 (6)*	$[M+H]^+$	MS^2 457: 209.1529 (100) MS^3 457-209: 191.1423(100); 163.1474(8)

			-	455.1915 (100) *456.1951 (25)* *457.1975 (6)*	$[M-H]^-$	MS^2 455: 191.1093(100)
Feruoylquinic acid	3.97, 4.46, 5.10	$C_{17}H_{20}O_9$	+	369.1185 (100) *370.1228 (20)* *371.1248 (4)*	$[M+H]^+$	MS^2 369: 177.0540 (100); 145.0277(30)
			-	367.1027 (100) *368.1060 (20)* *369.1079 (4)*	$[M-H]^-$	MS^2 367: 191.0556(100); 173.0451(45)

[a] m/z italics are isotopes

n.a – not available

Table S1. Results of HRMS untargeted screening: selection of peaks characterized by high intensity in positive and negative ionization modes. Comparison of MS/MS spectra performed with mzCloud database.

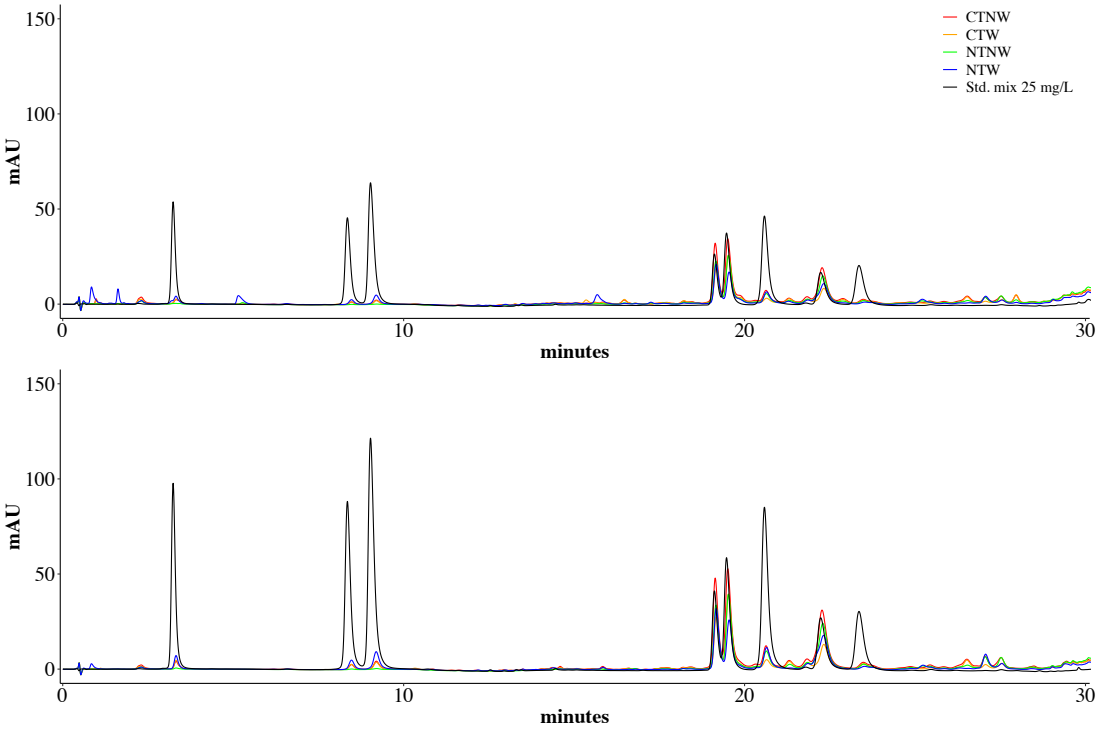

Figure S1. Representative chromatograms of standards solution and samples at (A) 280 nm and (B) 330 nm. (1): NCA, (2): CCA; (3) CA; (4): 3,5-DCQA; (5): Q-3-O-Gal; (6): Q-3-O-Gly; (7): Q-3-O-MG; (8): K-3-O-Gly.

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10. Conclusions

10.1. Agriculture sustainability meets human health

The results obtained in my PhD project support the role of semi-domesticated crops or neglected species in a framework of sustainable agriculture, to improve food production in the very next future both in western and developing countries.

Cowpea (*V. unguiculata*) is a performant legume crop which has already captured the attention of nutritionists and farmers due to its relevant properties not only at the nutritional level, but also in terms of prevention and ability to resist against a wide range of stressors. Many other legume crops share similar traits and therefore, the implementation of such kinds of crops among the most common dietary patterns deserves to be widely encouraged. Despite the well-documented health benefits of legumes consumption, their actual intake remains low (Polak et al., 2015). The concrete transition to a more sustainable diet richer in legumes requires a substantial change of the typical "western" dietary habits and food choices (also supported by industrial stakeholders), as well as suitable strategies to enhance legumes cultivation, distribution and consumption (Wolk, 2017). This aspect underlines how the concept of sustainability in the modern food systems is strictly related to the food and human health policies. Legumes well respond to both of these social-economic and political needs.

In this thesis, I demonstrated that *V. unguiculata* shows many environmental sustainability advantages such as i) low agrochemical demand (fertilizers); ii) ability to grow in harsh conditions like drought and by means of conservation agriculture (i.e., reduced or minimum tillage, cover crops maintenance); iii) adaptability to different climatic conditions of different continents and iv) suitability to be involved

in intercropping approaches with common cash crops, such as maize or wheat, as observed in Tanzania, with a consequent reduction of soil consumption, environmental pressure and plant damages. Moreover, yield performances of this species are good both in intensive and conservation management, without affecting its nutritional value. These features, combined with the tendency to reduce soil disturbance and to promote organic agriculture, should undoubtedly favor the spread of legume cultivation in different countries. I also underline that these features do not occur in many staple legume crops where the ecological footprint is higher than cowpea.

However, agronomical data are not sufficient to improve the consumption of these neglected African species and their diffusion worldwide. Social, economic and health levers are also needed to enhance the consumption of legumes (major and minor crops) in human diet. Since health issues are increasingly intertwined with food production systems and food consumer perception, they could act as drivers to select the most promising legumes, to enhance local production and to improve food distribution and consumption strategies.

Given these assumptions, an in-depth study of the micronutrients occurring in legume species (seeds and leaves), could suggest new forms of consumption, paving the way for food supplements and fortified foods, as well as to propose a circular economy process to valorize their by-products for human health purposes. Furthermore, the identification of bioactive compounds with beneficial or even therapeutic functions represents a lever of considerable importance also in relation to well-being and disease prevention, especially for the most vulnerable population groups (e.g., the elder population). The advantage of studying plant extracts compared to single isolated compounds or synthetic products lies in the fact that

phytocomplexes can exert a wider panel of bioactivities due to the multiple actions that different compounds may carry out.

10.2. Vigna unguiculata *as a preventive agent against cancer and neurodegeneration*

Bioprospecting analyses performed on *V. unguiculata* extracts suggested that this species is a source of peptides and small proteins displaying significant roles as health-enhancing compounds. Many studies showed that legumes proteins (mainly albumins and globulins), once digested, are able to gain a wide array of nutraceutical properties, acting as anti-inflammatory, anti-oxidants and also cancer preventive agents (De Fatima Garcia et al., 2020; Gonzalez-Montoya et al., 2018). My study focused on a Bowman-Birk trypsin and chymotrypsin inhibitor (BBI), composed of 107 amino acids with a molecular weight of 8-10 kDa. The data obtained in my experimental work suggest that BBI is a promising anticancer agent, able to slow down colon cancer cells proliferation and to interfere with cancer cells growth signals. Moreover, due to its resistance to harsh environmental conditions, such as the low pH of the stomach or the high temperatures which legumes are subjected to during boiling processes, BBI is very likely to join the target tissues. These features make this inhibitor a highly available peptide, able to exert effective cancer preventive activities. Moreover, other healthy relevant properties were attributed within this PhD work to the consumption of *V. unguiculata*. Aging and age-related disorders are among the most affecting disturbances arising during the elderly and diet is a pivotal element to counteract the outbreak of age-related pathologies. My results showed that *V. unguiculata* extracts are able to interfere with the negative effects caused by synucleinopathies. The responsible for such

kind of bioactivities were not deepened in the work, but some evidences suggested that a role may be played by protein fractions of high molecular weight (~80 kDa) belonging to the family of β-conglycinins, even though a synergistic actions among multiple components of the phytocomplex cannot be excluded. Interestingly, these properties were displayed not only *in vitro* but also *in vivo*, meaning that the compounds responsible for these bioactivities are able to act also in the complexity of an entire living organism.

10.3 Indigenous leafy vegetables as a source of micronutrients

Leafy vegetables such as the jute mallow (*C. olitorius*) were found to be characterized by peculiar traits, especially regarding the content of bioactive compounds. In this thesis, I mainly focused on the variation of antioxidant compounds and their health benefits, but a wider array of micronutrients already occurs in leaves tissues. Conversely to what found in the case of *V. unguiculata*, the experiments conducted on *C. olitorius* did not highlight a specific compound as responsible for the selective cytotoxic properties against the cancer cell line Caco-2, but it is the whole composition of the leafy polyphenolic fraction that is able to trigger such kind of bioactivity. This evidence brings the theme of the biological complexity existing at the metabolic level in healthy and cancer cells. The progress in the field of metabolomics, associated with advanced statistical techniques will be pivotal to better clarify which are the most active compounds and/or fractions of a phytocomplex. The most intriguing aspect is represented by the view-point change while addressing such kinds of studies, that is to consider the synergistic action promoted by a complex of different compounds that may result in nutraceutical properties, such as the antioxidant or the cancer preventive ones. This is in line with

some current pharmacological strategies aimed at identifying therapies based on a panel of beneficial compounds to reduce the incidence drug-resistance phenomena.

A more ecological aspect dealing with leafy vegetables concerns the sustainability of their cultivation. Generally, plants bearing large leaves show high transpiration levels and therefore are high-water demanding. This aspect limits the diffusion of species with a high leafy surface to the areas of the planets where there is high water availability. Climate change risks to notably reduce the diffusion of such species in many areas of the world. In this PhD thesis, however, I showed that there are leafy vegetables highly productive even under water stress regime and if managed with a conservation agriculture approach.

Further studies targeting other compounds are still ongoing, such as the comparison in the folic acid and derivatives content between some leafy crops (staple and indigenous) originating from East Africa (Kenya and Tanzania) to extend this nutritional investigation to the micronutrient composition of AIVs.

10.4. *Agrifood systems: the 'one health' concept*

On the whole, to join sustainability in agriculture it is important to consider not only the relationships existing between soil, water and plants, but also to look at the interactions that plants establish with the environment (e.g., considering micro-organisms and insects) at the ecosystem scale. In the Anthropocene era, humans are the main responsible for ecosystems alterations but, in a framework of sustainability, also in the light of the exponential growth increase faced by the human population, it is pivotal to understand and respect these complex interaction networks. The agriculture of the future should not be focused only on

the identification of more resistant crops or less impacting agriculture technologies. The time has come to have a close insight into the complex communication systems that biodiversity has developed. In this context, phytochemicals are not only compounds able to interact with healthy and cancer cells receptors, but also communication signals developed by plants to interact with the environment, since at least 300 millions years. One of the most intriguing aspects dealing with the study of phytochemistry is to assess which are the main modulator signals of these compounds and their elicitation mechanisms. In the last years, great interest has been devoted to the relationships occurring between plants and their pollinators. Pollinators are pivotal to guarantee plant reproduction and the fertilization is a crucial event which the majority of the agriculture sector is based on.

It is also important to understand the role of pollinators to promote the production of specific secondary compounds, useful for their nutritional requirements. Based on the quality of the rewards (e.g., the nectar) or the pollen they produce, pollinators may vary their health and fitness. The current decline of pollinators worldwide may be partly explained by the worsening of the resource quality provided by plants to insects as a consequence of the alteration of environmental balances.

Very recently, meta-analysis data showed a relationship between pollination mediated by insects and human NCDs occurrence (Smith et al., 2015). This underlines the complex interaction network existing among different ecosystem actors. Given these assumptions, in the last part of my PhD thesis, with the complicity of the COVID-19 lockdown, I focused on the currently known relationships existing between phytochemicals and pollinators diet and many researches go toward the direction of identifying secondary metabolites

responsible for beneficial healthy properties to pollinators. For instance, *p*-coumaric acid is a phenolic compound usually occurring in pollen and able to activate the detoxifying defence mechanisms of honeybee larvae against xenobiotics such as pesticides and to increase their chronological lifespan, delaying cellular aging processes (Mao et al., 2013). Understanding how these healthy promoting compounds may be elicited could be a key aspect to slow down or also to reverse the current decline faced by pollinators communities. Furthermore, recent studies highlighted also the role of pollination in improving the quality of fruit (Lama et al., 2020) due to an elicitation of organic acids metabolism, responsible for a better fruit taste and palatability. Interestingly, organic acids such as citrate and malate were found to be higher in pollinated fruit than in parthenocarpic ones and this opens new scenarios towards the evaluation of the role of allogamy promoted by pollinators in improving the nutritional quality of fruit and seeds and therefore on human diet.

10.5. *Rethinking the Agrifood systems in the urban context*

Considering that human life is even more concentrated within big cities, to foster human health it is also important to promote policy actions aimed at encouraging urban agro-ecological activities. The growing urbanization is forcing even more natural lands exploitation for agronomic purposes in order to satisfy the increasing food demand. However, a recent study highlighted that the increasing amount of intensive agronomic exploited lands is risking to pose severe nutritional deficiencies to pollinators, due for instance to a worrying decrease in the pollen protein content of the foraged plants within highly exploited agricultural environments (Donkersley et al., 2014). The challenge of the agriculture of the future needs therefore to be

played in new contexts, for instance by including cities in proximity agriculture projects, to reduce the pressure of agricultural lands on natural landscapes. To address this goal, we need to know if a highly impacted environment, such as that of cities, may guarantee pollinators adequate nutritional resources able to support and maintain the ecosystem service. At the same time, the proximity agricultural strategy and the involvement of citizens in the agri-food chain could enhance the awareness that food production, distribution and consumption are key elements of modern days lifestyle to avoid the risk of malnutrition and to promote human wellbeing.

11. Appendix

1. Daniela Scaccabarozzi & Lorenzo Guzzetti, Ryan D. Phyllips, Lynne Milne, Nicola Tommasi, Salvatore Cozzolino, Kingsley W. Dixon (2020). Ecological factors driving pollination success in an orchid that mimics a range of Fabaceae. *Bot J Lin Soc*, *194*(2), 253-269.

2. Daniela Scaccabarozzi, Salvatore Cozzolino, Lorenzo Guzzetti, Andrea Galimberti, Lynne Milne, Kingsley W. Dixon, Ryan D. Phillips (2018). Masquerading as pea plants: behavioural and morphological evidence for mimicry of multiple models in an Australian orchid. *Annals of Botany XX*, 1-13.

Contribution: in these two works I managed the analyses of field data to identify the batesian mimicry patterns of two Australian orchids belonging to the genus *Diuris*. The statistical analysis was carried out through mixed effect regression models (frequentist approach) to identify both linear and non-linear relationships between tested variables.

3. Chiara Magoni, Ilaria Bruni, Lorenzo Guzzetti, Mario Dell'Agli, Enrico Sangiovanni, Stefano Piazza, Maria Elena Regonesi, Mariateresa Maldini, Roberto Spezzano, Donatella Caruso, Massimo Labra (2018). Valorizing coffee pulp by-products as anti-inflammatory ingredient of food supplements acting on IL-8 release. *Food Res Int, 112,* 129-135.

Contribution: in this work I supported the phytochemical investigation of coffee pulps by products and I analyzed chemical data for the set-up of antioxidants extraction protocols.

4. Paolo Biella, Nicola Tommasi, Asma Akter, Lorenzo Guzzetti, Jan Kekla, Anna Sandionigi, Massimo Labra, Andrea Galimberti (2019). Foraging strategies are maintained despite workforce reduction: a multidisciplinary survey on the pollen collected by a social pollinator. *Plos One*, *14*(11).

Contribution: in this work I supported the analysis of data coming from an experiment on bumble bees foraging behavior to verify the impact of colony halving on the variation of resource preferences and collection.

5. Andrea Galimberti, Maurizio Casiraghi, Ilaria Bruni, Lorenzo Guzzetti, Pierluigi Cortis, Nadia Berterame, Massimo Labra (2019). From DNA barcoding to personalized nutrition: the evolution of food traceability. *Current Opinion in Food Science*, *28*, 41-48.

Contribution: in this work I contributed to data visualization by producing graphs showing the increasing trend along the last 15 years in the usage of DNA barcoding techniques in the field of food analysis and traceability.

6. Valerio Mezzasalma, Anna Sandionigi, Lorenzo Guzzetti, Andrea Galimberti, Maria S. Grando, Javier Tardaguila, Massimo Labra (2018).

Geographical and cultivar features differentiate grape microbiota in Northern Italy and Spain vineyards. *Front Microbiol*, *9*, 946, doi:

Contribution: in this work I collaborated to the multivariate analysis of data (PCoA and PERMANOVA) coming from the microbial assessment of grapes from different geographical origin to understand which are the main parameters influencing the microbial communities of grapes and – consequently - the quality of grapes derived products.

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