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Research Article

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Effects of chemical composition and genetic control of tocol on Anticancer activity of seeds of Grape (*Vitis vinifera* L), date palm (*Phoenix dactylifera* L.), pomegranate (*Punica granatum* L .) and olive (*Olea europea* L.)

Ashwag A.A. Mohamed Nour*, Mohamed T. Yousif

Department of Biotechnology - Faculty of Agriculture Abunaama- University of Sinnar- Sudan
*Corresponding Auhtor: longingshr@yahoo.com

Abstract A study aimed at studying antitumor activity of seed extracts of olive (*Olea europea* L.), date palm (*Phoenix dactylifera* L.), pomegranate (*Punica granatum* L.), and grape (*Vitis vinifera* L) and to correlate their anticancer activity with similarity and dis-similarity with respect to fatty acids composition and phenolic compounds as well as studying similarity in tocols content, their genetic control and gene expression. The potato disc bioassay technique was used to detect for antitumor activity and thin layer chromatography (TLC) was used to identify active components in the crude extract (oil) of the four plant species. Whereas, bioinformatics tools were used to identify similarity and dissimilarity in the phenol, tocol (tocopherol) concentration and genetic control of tocol expression as well as similarity in tocol genes of the four plant and their clustering. Bioassay tests indicated antitumor activity of plant species with grape and date (100%Inhibition) ranked first followed by pomegranate (60%) and olive (45-50%) also possessed considerable similarity in chemical constituent were performed using (TLC) and biological activity as antitumor. Results revealed variation with respect to fatty acid composition and amount of phenols and tocol expressed inside seed cells of the different plant used. Concerning the tocol gene construction, close similarity was found among tocols of the different species detected in gene domains constructs and phylo-genetic analysis. Correlation of antitumor activity with phenol and tocol content indicated a positive correlation; whereas close similarity in the tocol amino acid sequence and clustering analysis indicates similarity in the gene construct.

Keywords tocol, phenol, chemical constituent, similarity, bioinformatics tools

Introduction

Natural antioxidant of plants could be extracted in the form of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids [1]. Antioxidants that are reducing agents can also act as pro-oxidants [2]. All of these antioxidants are different in their effectiveness. Some dried fruits such as pomegranate, grapes, olives, ginger and figs showed significantly higher phenolic content than the fresh ones [3].

The rank of the amount of phenolics in fresh fruits and plant samples was as follows:

red grapes > black olives > green olives > pomegranate > ginger > garlic > date fruits > red onion >white graps >figs > gourd > snake cucumber> banana > lentils > wheat > cucumber. Tocopherols are a class of naturally occurring chemical compounds related to vitamin E and act as antioxidant agents. Anti oxidant activity of different tocopherols depends on temperature, lipid composition, physical state and tocopherol concentration [4]. Alpha-Tocopherol, used as calibration standard, showed the highest ferric reducing antioxidant power. Greater ring methyl substitution not only led to an increase of scavenging activity against the stable 2,2-diphenyl-1-picrylhydrazyl radical, but also to a decrease in oxygen radical absorbance capacity [5]. The tocopherol's strength arranged as δ>γ>β>α [6]. Both tocopherols and tocotrienols with these eight vitamers (α-, β-, γ-, and δ-tocopherols and tocotrienols, respectively) are also known collectively as vitamin E [7].

Grape contains several polyphenols, among them the dominant resveratrol (a polyphenol antioxidant), which may explain the health benefits of grapes [8].

A Protection of the genome through antioxidant actions may be a general function of resveratrol [9]. Olives (*Olea europaea*) particularly the black variety was in the extremely high group with pomegranate and grape fruits in its ability to scavenge free radical as compared to the two strong antioxidant standards; vitamins C and E. Several polyphenol antioxidants were isolated from olive oil. Among the most abundant are; 3,4 dihydroxyphenyl-ethanol and phydroxyphenylethanol. Both compounds increased the intracellular concentration of Ca^{2+} in a dose dependent manner [10]. Date fruits (*Phooenix dactylifera*) showed high radical scavenging activity with a moderate level of polyphenols present. The phenolic profile of date fruits revealed the presence of mainly Cinnamic acids, flavonoid glycosides and flavonols in addition to gallic acid, caffeic acid and pcoumaric acid. Anthocyanins and steroids were detected only in fresh dates as well as several enzymes, such as phytase, invertase and peroxidase have been isolated as well [11].

0.8-2.2 mg/ml of fresh doses of date fruitsare capable to scavenge 50% or more of superoxide, hydroxyl and lipid peroxides radicals in a dose dependent fashion [12].

Bioassay methods offer special advantages in establishing the biological purposes (antitumor, antibacterial, antioxidant and phytotoxic properties *etc.*). Bioassay is the preliminary step in drug discovery which allow the screening of biological and synthetic bioactive compounds [13]. Potato disc assay was shown to be useful for checking known and novel antitumor molecules' properties. This bioassay is based on *Agrobacterium tumefaciens* infection on potato disc [14]. The validity for the use of such assay is that the tumorogenic mechanism initiated in plant tissues by *A. tumefaciens* is in many ways similar to that of animals [15]. In fact, Kempf, *et al.* [16] showed that *Bartonella henselae*, a bacterium causing tumor in human shares a similar pathogenicity strategy, with plant pathogens *A. tumefaciens*. Several studies are finding numerous and significant areas of similarity among the mechanisms used by bacterial pathogens of plants and humans. These similarities include the use of common toxins, secretion system, adhesion mechanism, invasion and regulation [17]. This method is used during the last 15 years and it appears to be adaptable to the purpose of standardization or quality control of bioactive compounds [18]. It has been shown that the inhibition ability of crown gall formation by *A. tumefaciens* on potato discs and subsequent growth was in good correlation with compounds and extracts, which are statistically much more predictive of *in vivo* and *in vitro* anti-leukemic activity [13]. In Sudan, identification of the indigenious strain of A. tumefaciens SDB 0012 [19] provides a room for conducting bioassay tests for anti-tumor activity of natural products, in which Sudan is endowed with very high genetic variability.

A comparative study was carried out at University of Gezira- Faculty of Engineering and Technology-Bioscience and Biotechnology Center in 2013. This study aimed at studying antitumor activity of seed extracts ofolive (*Olea europea* L.), date palm (*Phoenix dactylifera* L.), pomegranate (*Punica granatum*), and grape (*Vitis vinifera* L) and extracts and to correlate their anticancer activity with similarity and dis-similarity of the four plant species in fatty acids composition and phenolic compounds as well as studying similarity in tocols content and their genetic control and gene expression in the four plant species. The detailed objectives of this study were included:

1. Computer survey to collect information on seed composition of the four selected plant species with regards to mineral content, oil composition and phenol compounds.
2. To assess for anti-tumor activity of seed extracts of the selected plant species, using the potato disc bioassay techniques.
3. Separation and alignment of chemical components of seeds of the selected plant species using TLC technique.
4. To study similar and dis-similarity in tocol genes of the four plant species, using bioinformatics tools.

Materials and Methods

This study was conducted at the Bioscience and Biotechnology Center of Faculty of Engineering and Technology-University of Gezira, Sudan in 2013-2014. It aimed at studying similarity in anti-tumor activity of four plant extracts and to correlate their anticancer activity with similarity and dis-similarity of the four plant species in fatty acids composition, mineral content and phenolic compounds as well as studying similarity in tocols content and their genetic control and gene expression.

Extraction

The extraction was carried out using three different solvents, i.e. methanol, Hexane and Ethyl Acetate to extract polar, non-polar and medium polar compound. Ten grams of fruit seeds were weighted after drying under shade and ground in a mortar. The fruit seed was extracted with 150 ml of absolute n-hexane, methanol, and ethyl acetate respectively using shaking for few mint's at room temperature for 27 hours, the filtrate was concentrated and used later for further analysis TLC.

Bioassay techniques: The potato disc bioassay technique was conducted following McLaughlin and Rogres [20]. Experiments were conducted using the indigenous strain of *Agrobacterium tumefaciens* [19]. Each treatment in the different experiments was represented by 3 Petri dish per sample. Lugol's reagent stains the starch in the potato tissue a dark blue to dark brown color, but the tumor produced by *Agrobacterium tumefaciens* will not take up the stain, and appear creamy to orange [20]. The stained potato disk were viewed under a dissecting microscope and the tumor were cont.

Thin Layer Chromatography

TLC was used to separate individual chemical constituents of the fruits seeds extract. From a diverse range of solvent systems used for performing TLC, Hexane /Diethyl ether /formic acid (80:20:2), Benzene /Ethanol /Ammonium hydroxide (90:10:1) and Petroleum ether /Chloroform /Ethyl acetate (2:2:1) which presented good separation of the band on TLC plate with good quality resolution.

This study was carried out to compare the chemical separated components according to: Fixed oils extracted from fruits seeds samples were subjected to TLC separation for their Oils constituents as shown in Fig (1 & 2).

Oil components prepared by TLC technique for fruits seeds samples were prepared from seeds by cold extraction method, separated by TLC solvent system Hexan /Diethyl ether /formic acid (80:20:2), and identified by Ferric Chloride ($FeCl_3.6H_2O$) 50 mg was added to 90ml distill water ml followed by 5ml acetic acid and 5 ml concentrated sulfuric acid. In Fig (1**),** the same number of TLC-separated compounds was obtained by samples which represented the number of five separated compounds similar in (R_f and color) in all samples.

Bio-informatics tools were used for tocolprotein alignment (NCBI-BLAST), phylogenetic analysis of tocol genes of the four plant species (NCBI-Clustal W. Program) and single nucleotide polymorphism using the next generation sequencing technology (Bio Edit Sequencing Protein Software).

Results and Discussion

Fatty acid composition of grape, date, olive and pomegranate showed polymorphism in contained fatty acid palimitic, oleic, linoleic, palmitoleic, steariclinolenic, lauric, punicic, arachidic and gadoleic with the former three fatty acids remain conserved among fatty acids of the four plant species (Table 1). Olive contains eight of the ten fatty acids mentioned, pomegranate (five), date (four) and grape (six). Lauric and punicic missed in olive and grape, arachidic and gadoleic missed in grape, date and pomegranate. Whereas, palmitoleic and linolenic missed in date and pomegranate. In addition lauric missed in pomegranate. Antitumor activity tests using potato disc bioassay showed 100% inhibition in grape and date palm methanol extracts, 60% inhibition in pomegranate and 45-50% inhibtion in olive (Figure 1). The tumor cells were detected as yellow to orange colour cells on the potato discs while brown to dark colors indicated normal cells.

Antioxidants are chemicals that block the activity of other chemicals known as free radicals. Free radicals are highly reactive and have the potential to cause damage to cells, including damage that may lead to cancer [21].

The free radical scavenging activity (antioxidant activity) and the amount of total phenolics in plant samples showed a strong positive correlation when domenstrated in pomegranate, grape, olives, date fruits and ginger [3]. The red grape scored the highest amount of phenol content (150 mg GAE/g) followed by black olive (144.47 mg GAE/g), Pomegranate (76.93 mg GAE/g) and dates (47 mg GAE/g), as presented in Table 1. These findings when correlated with potato disc bioassay results obtained in this study showed no correlation between phenols content and anticancer activity in the four plant species and recommend more studies to correlate anti-cancer activity with phenol quality and phenol construction. Since dates scored less amount of phenol content compared to olives but as the same time it gave better results in the potato disc as anti-cancer activity. Oil content of grape seeds strongly depends on grape (*Vitis vinifera* L), family Vitaceae, variety, though the usual range is 10–16% of dry weight [22]. Compared to other commercial vegetable oil (Table 2), grape seed oil is the most similar oil with date seed oil in terms of total tocols content [7]. Although olive oil is one of the edible oil that contains high phenolic contents [23], review had shown that the total tocols content of date seed oil found by Nehdi *et al.* [24] is higher than that of olive oil. These findings might explain similarity in results obtained in the potato disc bioassay for grapes and date, ranked first, and the intermediate anticancer activity of olive crude extract (figure 1). If though, emphasizes the role of tocols contents in the anticancer activity. It also recommend elucidation of anticancer activity of Black raspberry seed oil* (2) since it contained the highest amount of tocol content (166.0 mg/100g) ,and soybean oil (99.9 mg/100g), as presented in table, compared to 52 .2 mg/100g ikn grapes and 51.5 mg/100g in dates.

A positive correlation between antioxidant activity and its phenolic content. In this study, grapes (*Vitis vinifera*) in general, and red grapes in particular, showed the highest radical scavenging activity followed by pomegranate (*Punica granatum* L)and by the two types of olives; green and black olives. These results were in aline with those obtained in anti-cancer activity since grape showed the highest anticancer activity followed by pomegranate and olive, which in-turn correlated anticancer activity with antioxidant activity and phenol content. Concerning TLC of crude extract of the four plant species using hexane. Five component presented in the four species, as presented in (Figure 2), at the top of the plates. They were found be similar in color and Rf. According to these five components were equivalent to cholesteryl esters, free fatty acids, a,a-diglycerides and α-, β-diglycerides from the top of the plate to the bottom. The three components triglycerides, free fatty acids and α-, β-diglycerides were found to have antioxidant effects [25], which might approve the antioxidant effects of the four plant species.

Alignment of protein domains (five domains) of grape, date and olive showed similarity in the domain structure of grape, date palm and olives with the same profile of domains (Figure 3). Clustering analysis of the tocol aminoacids showed eight evolved groups of grape tocols (20 tocol genes). With high similarity among tocols 1,5,10,11, and tocol 13 to which olive tocol shares the same evolution and similarity as one cluster groups and other tocol as another cluster group, as presented in Figure 4. Date palm tocol sequence was found to be very close to tocol 1 and tocols 14-20 of grape. These findings emphasize the important role of grape tocol as antitumor agents since it contains 20 different tocols with close similarity of tocol 1 to date palsm and tocol 5 to olive tocol. Cluster analysis indicated stability of the tocol gene when correlated with similarities in protein domains of grape, date palm and olive.

Conclusions

Results obtained in this study showed variation in fatty acid composition of crude extracts (oil) of grape, date palsm, pomegranate and olive, and phenole and tocol contents. On the other hand, the four tested plant species showed anticancer activities with grape and date plam (100% inhibition) ranked first followed by pomegranate (60% inhibition) and olive (45-50% inhibition). High similarity was found in protein domains (five domains) of grape, date and olive and close similarity of date and olive tocols were found with grape tocols. These findings concerning similarity and dis-similarity of parameters and those correlated with anti-tumor activity studied

Table 1: Fatty acid composition of grape, date palm, pomegranate and olive

Fatty acid	Palmitic *C16:**0	Palmitoleic C16:1	Stearic. C18:0	Oleic C18:1	Linoleic, C18:2	Linolenic C18:3	Lauric C12:0	Punicic C18:3	Arachidic, C 20:0	Gadoleic C 20:1
Grape	10,57	0,74	6,13	29,10	52,26	0,75	-	-	-	-
Date palm	10	-	-	50	19	-	10	-	-	-
Pomegranate	4.8	-	2.3	6.4	6.6	-	-	65.3	-	-
Olive	7.5-20	0.33.5	0.5-5.0	55.0-83.0	3.5-21	<1.0	-	-	<0.6	<0.4

*=Number of carbons in a given fatty acid; **= number of double bonds.

Table 2: Content of total phenolic content in wet extract**s**

Plant Material	mg GAE/g FW	
Red Grapes	150.69 ± 1.87	high
Black Olives	144.47 ± 0.94	
Green Olives	84.08 ± 3.18	
Pomegranate	76.93 ± 8.80	
Ginger	76.93 ± 2.06	
Garlic	61.44 ± 1.12	moderate
Dates	47.40 ±1.50	
Red Onions	34.03 ± 2.06	
White Grapes	33.50 ± 1.31	
Figs	29.26 ± 1.69	
Gourd	24.10 ± 2.25	
Snake Cucumber	15.63 ± 1.87	
Banana	14.04 ± 1.50	
Cucumber		
Red Grapes	1.32 ± 1.50	low

Table 3: Tocols contents of date seed oil and other vegetable oil (mg/100 g oil)

Samples	Total tocols (mg/100g)
Date seed oil (*P.Canariensis*)* (1)	51.5
Black raspberry seed oil* (2)	166.0
Soybean oil* (2)	99.9
Corn oil* (2)	61.1
Olive oil* (2)	28.0
Canola oil* (2)	27.0
Perilla oil* (2)	45.4
Grape seed oil* (2)	52.2

*1) Nehdi*et al.*, [25]; 2) Adhikari *et al.*, [7]

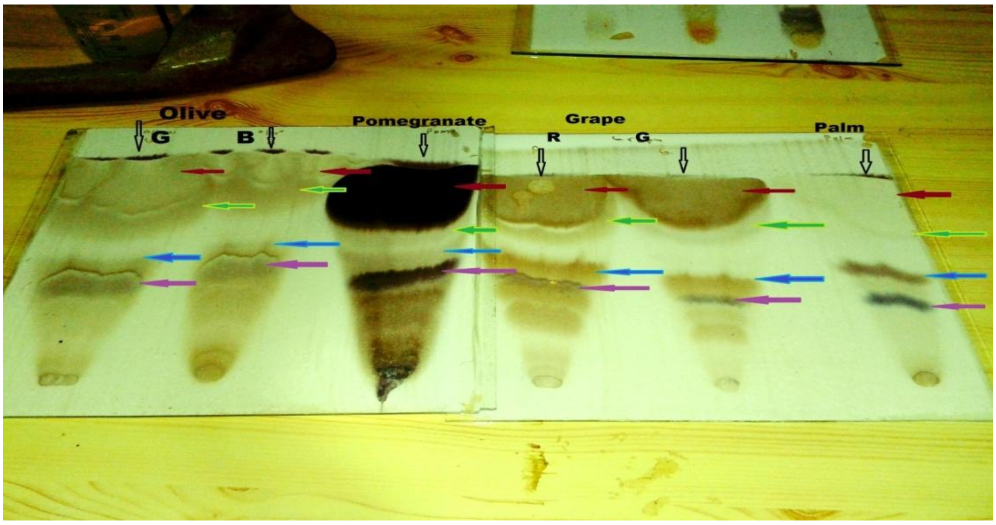
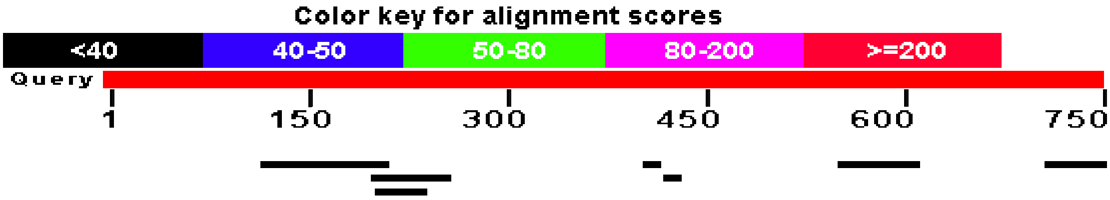
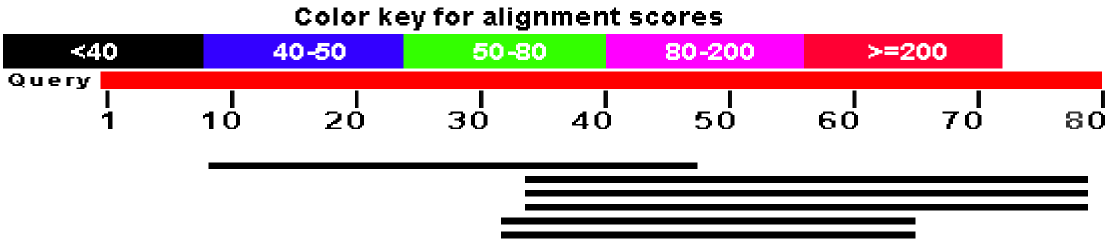


Figure 2: Oil extracted of crude extracts (oil) of seeds of date palm, grape, pomegranate and olive, using hexane as solvent

Grape



Date



Olive

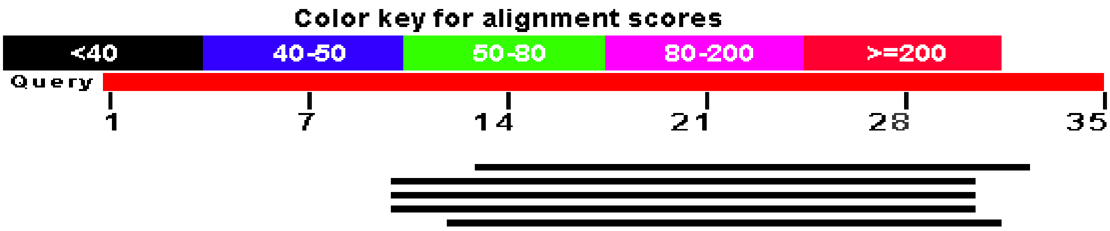


Figure 3: Similerty in tocol Domains of Grape, Date palm and Olive

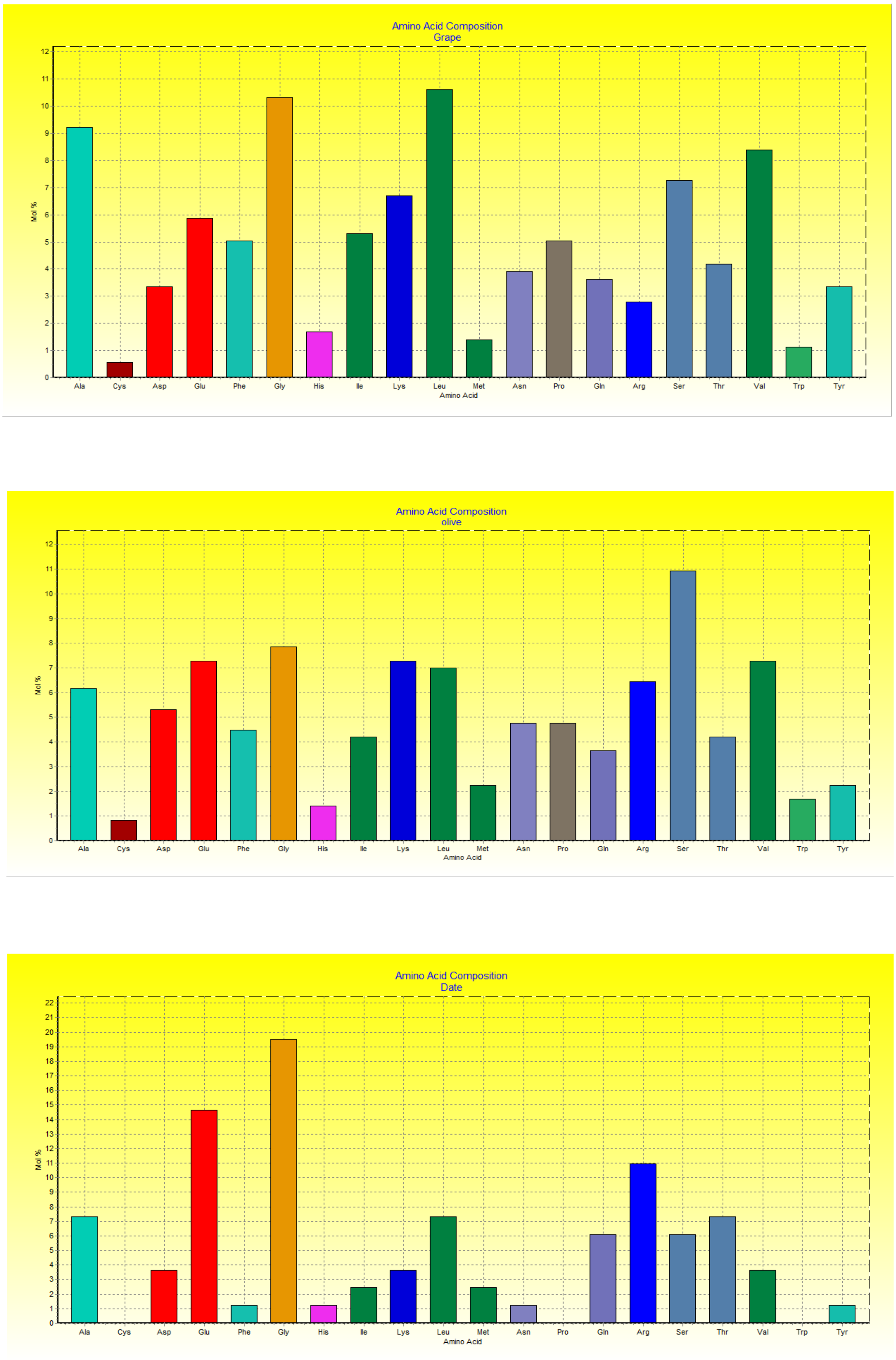


Figure 4: Cluster analysis of tocols genes of Grape ,Date and Olive(Using Bio Edit Sequence Program)

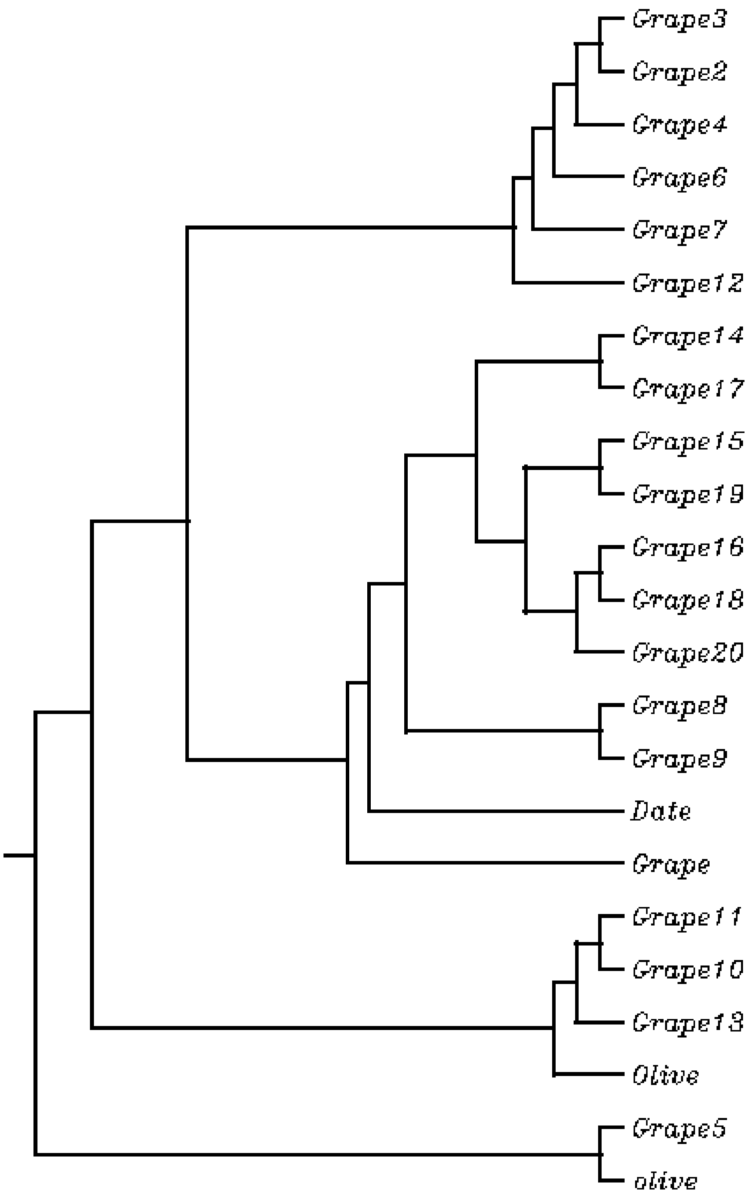


Figure 5: Phylogenetic tree of Tocols(Using CLustal W. Program)

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