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Biochemical and molecular characterization on 11 cultivars of *Coffea arabica* L

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Abstract
The present studies are interested the chemotaxonomy including the phytochemical and molecular screening of the 11 cultivars of *Coffea arabica* obtained from Jazan, KSA. Total alkaloids, terpenoids, flavonoids, saponins were assessed and compared. Alkaloids and flavonoids are assayed by the method of thin layer chromatography (TLC). 14 phenolic compounds assayed by high-performance liquid chromatography (HPLC) method. Seed protein diversity as reveled by variation in SDS-PAGE techniques been used as molecular methods. Statistical analysis of the phytochemical and the molecular data are analyzed separately and grouped using Minitab 17.0 statistical software. obtained statistical data resulted in two groups, the first includes cv. *jazani*, cv. *Yemeni*, cv. *Chinese*, cv. *Colombian*, cv. *Habshi*, cv. *Yafi*, cv. *Indian*, cv. *Kenya*n and cv. *Brutte*. The second group included the two cultivars of *Ethiobian Harar* and *Brazilian*. In the first group, three subgroups are recognized, cv. *Yafi*, cv. *Yameni* and cv. *Jazani*; the second subgroup included cv, *Chinese*, cv. *Habshi* and cv. *Colombian* meanwhile the third subgroup included the three cultivars of *Indian, Kenyan* and *Brutte.*

Keywords: *Coffea arabica L,* Biochemical analysis, TLC, HPLC, Seed Protein electrophoresis

1. Introduction
Genus *Coffea L* which related to family Rubiaceae has two species, *Coffea arabica* L. (coffee) and *Coffea canephora* Pierre (Robusta) (Raina *et al*., 1998 and Lashermes, *et al*., 1999) [15, 8]. *C. arabica* which is indigenous to Ethiopia and comprises about 73% of world coffee production due to its superior quality (Orozco-Castillo *et al.* 1994) [12]. The ethanolic plant extracts consider the popular methods in determination of flavonoids, phenolics, terpenoids, saponins and alkaloids when subjected to determine the qualitative chemical tests. Different phenolic compounds have a significant role in differentiation between different plant taxa (Belitz *et al*., 2008) [2]. Monteiro and Farah (2012) [10] have evaluated the chlorogenic acids (CGA) content and profile in economically important Brazilian cultivars of coffee. Aluminum chloride ($AlCl_3$) colorimetric is suitable method for the flavonoid determination (Sudarshan *et al*., 2014) [17]. The protein electrophoretic separations now have an established place in modern chemotaxonomic practice (Harborne and Turner, 1984) [6]. Electrophoretic technique considers a good tool for taxonomic purposes not only for taxonomists, but also to biochemists (Gottlieb, 1978) [5]. Rogers *et al.* (1999) [16] mentioned that, legumin like proteins are the main storage protein in the most coffee species. Yuffá *et al*., 1994 and Rogers, *et al*., 1999 [20, 16] stated that, there are qualitative and quantitative differences in size and charge in the protein patterns between two species of *Coffea* under polyacrylamide gel electrophoresis. Sandra *et al*., 2001 [19] stated that, no marked difference in the banding profile of *coffea* cultivars, only marked differences were observed among species, or even among individuals of some species. The present work sought to the complete information on the phytochemical and molecular data and resolving the similarity and dissimilarity between the *Coffea arabica* cultivars.

2. Materials and Methods
Seeds of the eleven cultivars of *Coffea arabica* were obtained from Agricultural Research Station in Bani Malek of Jazan, KSA.

2.1. Phytochemical analysis
I- Total biochemical analysis
For estimation the total alkaloid, seed extracts were soaked in 80% ethanol dissolving in 20% acetic acid, filtered and evaporated to one-quarter of the original volume. Concentrated

ammonia solution was added to seed extracts until precipitation was completed Harborne (1973) [7]. For estimation of falvonoid, extracts are soaked in 80% aqueous methanol, filtrated, evaporated and weighted (Boham and Kocipai, 1974) [4]. Estimation of saponin, extracted seeds were soaked in 20 % ethanol, then transferred into separating funnel in which 20 mL of diethyl ether and shaken vigorously with about 30 mL on n-butanol. The combined n-butanol extracts were washed with 5% aqueous sodium chloride. Evaporated sample was dried and the saponin content was calculated (Obadoni and Ochuko, 2003) [13]. Total terpenoids carried out according to methods of Olayiwola (2013) [14].

2.2. A. Thin layer chromatography (TLC)

The dried alkaloid extract dissolved in chloroform: methanol (95:5) according to methods of (Olayiwola, 2013) [14]. The dissolving extracts subjected to thin layer chromatography which performed on pre-coated silica gel plates (DC-Alufolien 60 F254, Merck). The chromatogram of the alkaloids was observed under UV before and after sprayed with trichloro acetic acid as a reagent. Dried flavonoid extract was subjected to thin layer after dissolving the extract in the eluting system of ethyl acetate: methanol: water (30: 5: 4). The chromatogram of the flavonoid was observed under UV before and after exposure to ammonia and sprayed with $AlCl_3$ as a phenolic reagent.

2.3. B. HPLC analysis of flavonoid

For extraction and identification of phenolic acids by HPLC (Amarowicz *et al.* 1995) [1]. The samples were extracted according to the method outlined by Ben-Hammouda *et al.* (1995) [3]. Identification of individual phenolic compounds of the plant samples was performed on a Hewlett-Packard HPLC (Model 1100), using a hypersil C_{18} reversed-phase column (250 x 4.5 mm) with 5 µm particle size. Injection by means of a Rheodyne injection valve (Model - 7125) with 50 µl fixed loop was used.

II-Seed protein methods

Characterization of seed protein fractions are carried out using one dimential sodium dodecyl sulphate (SDS-PAGE). Preparation and running of the gel were carried out according to Stegeman *et al*., (1988) [18] and Luth (1992) [9]. The gel was stained with comassie brilliant blue stain R-250. Bands were determined and scanned using Hoefer scanning densitometer GS 300. Protein gel bands scanned and photographed.

III-Statistical analysis

Statistical Analysis of the identified data was carried out by Multivariate Cluster analysis using Minitab *17.0* Statistical software.

3. Results and Discussion

I- Phytochemical data

All the eleven cultivars contain alkaloids, flavonoids and terpenoids. saponins were detected in all the cultivars except cv. *Chinese*, cv. *Ethiobian*, cv. *Yafi* and cv. *Kenyan*. The highest percentage of alkaloid content (0.290%) was noticed in cv. *jazani* while the lowest one was recorded in *Indian* cultivar. On the other hand, the highest values of flavonoid contents (1.230 and 1.067) was noticed in cv. *Ethiobian Harar* and *Brazilian*, respectively. Also, the value of 0.989 was recorded in cv. *Colombian.* The highest terpenoid values of 0.908 and 0.941 are recorded in *Yemeni* and *Kenyan* cultivars, respectively (Table 1).

Table 1: Results of the totals phytochemical analysis of the *Coffea* cultivars

Constituents / Cultivars	Alkaloid	Flavonoid	Terpenoid	Saponin
Jazani	0.290	0.859	0.802	0.708
Yemeni	0.117	0.012	0.908	--
Chinese	0.114	0.132	0.631	0.420
Colombian	0.166	0.986	0.840	0.551
Habshi	0.021	0.809	0.680	--
Yafi	0.160	0.902	0.731	--
Ethiobian Harar	0.156	1.230	0.361	0.708
Brazilian	0.192	1.067	0.730	0.860
Indian	0.016	0.344	0.880	0.254
Kenyan	0.173	0.783	0.941	0.422
Brutte	0.260	0.453	0.820	--

To confirm the presence of alkaloids and flavonoid in the cultivars, thin layer chromatography (TLC) was performed. Observed proximate chemical composition of *Coffea arabica.* The analyzing data of 11 cultivars showed that, RF of alkaloid constituents ranged between 0.25- 0.228 %. The minimum value was estimated in cv. *Brutte* meanwhile the maximum value was estimated in cv. *Yafi* cultivars. On the other hand, two spots are noticed in all studied cultivars except cv. *Brazilian* which has three spots (Table 2).

Table 2: Results of the alkaloid contents in the cultivars using thin layer chromatography (TLC)

Data / Cultivars	Spot No.	Color Characterization		RF 1	RF 2	RF 3
		Visual	U V Light			
Jazani	Spot -2	Pale Brown	Purple	0.19	0.26	--
Yemeni	Spot -2	Brown	Purple	0.18	0,25	--
Chinese	Spot -2	Brown	Purple	0.17	0,26	--
Colombian	Spot -2	Brown	Purple	0.16	0,26	--
Habshi	Spot -2	Brown	Purple	0.18	0,27	--
Yafi	Spot -2	Brown	Purple	0.15	0,28	--
Ethiobian Harar	Spot -2	Brown	Purple	0.15	0.25	--
Brazilian	Spot -3	Brown	Purple	0.17	0.28	0.50
Indian	Spot -2	Pale Brown	Purple	0.18	0,25	--
Kenyan	Spot - 2	Brown	Purple	0.18	0,26	--
Brutte	Spot -2	Brown	Purple	0.18	0,25	--

Data of Table 3 revealed that, RF of flavonoid ranged between 5.1 % as a minimum value which noticed in cv. *Ethiobian Harar* and *Brutte* cultivars to 9.7 % as a highest one which in turn noticed in cultivar *Jazani*. Three spots are noticed in *Yafi, Ethiobian Harar* and *Brazilian cultivars,* while one spot are watched in *Habshi and Kenyan.* Two spots are appeared in the remainders . TLC plate analysis showed that, all the studied cultivars gave a positive reaction for the flavonoid compounds and gave different brown because the aluminium chloride ($AlCl_3$) served as a developer to make visible the secondary metabolite on the TLC plate (Fig. 1).

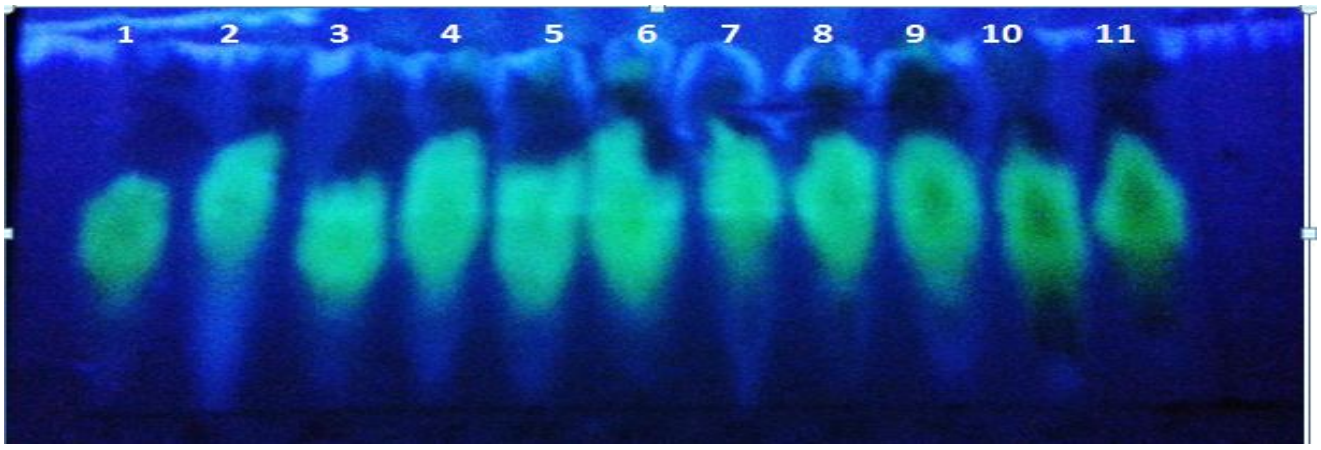

Fig. 1: TLC of ethanolic seed extract of flavonoid in *Coffea arabica* cultivars detected by UV light; *1- jazani, 2- Yemeni, 3-Chinese, 4- Colombian, 5- Habshi, 6- Yafi, 7- Ethiobian Harar, 8- Brazilian, 9- Indian, 10- Kenyan, 11- Brutte*

Table 3: Results of the flavonoid contents in the cultivars using thin layer chromatography (TLC)

Data / Cultivars		Color Characterization		RF 1	RF 2	RF 3
		Visual	U V Light			
jazani	Spot -2	Brown	Yellow	5.2	9.7	--
Yemeni	Spot -2	Brown	Yellow	4.9	9.5	--
Chinese	Spot -2	Pale Brown	Pale Yellow	5.6	9.5	--
Colombian	Spot -2	Brown	Yellow	5.8	9.4	--
Habshi	Spot -1	Brown	Yellow	5.7	--	--
Yafi	Spot -3	Brown	Yellow	5.2	6.7	9.4
Ethiobian Harar	Spot -3	Brown	Yellow	5.1	7.6	9.2
Brazilian	Spot -3	Dark Brown	Yellow	5.3	8.2	9.5
Indian	Spot -2	Brown	Yellow	4.6	7.3	--
Kenyan	Spot - 1	Brown	Yellow	5.7	--	--
Brutte	Spot -2	Brown	Yellow	5.1	7.7	--

3.1. HPLC results

Qualitative and quantitative estimation for the phenolic compounds of *Coffea* cultivars was determined by HPLC, each compound was identified using authentic pattern and the concentration was achieved as relative percent. The separated and identified phenolic compounds were caffeic acid, ferulic acid, sinapic acid, gallic acid, protocatechuic acid, *p*-hydroxy benzoic, *p*-coumaric, phenol, o-coumaric, chlorogenic acid; while 4 unknown compounds also separated as in Table (4).

Table 4: Relative percent of phenolic compounds of *Coffee arabica* cultivars using HPLC

Cultivars / Constituents	*Cult. jazani*	*Cult. Chinese*	*Cult. Yemeni*	*Cult. Colomb.*	*Cult. Habshi*	*Cult Yafi*	*Cult. Ethiobian Harar*	*Cult. Brazilian*	*Cult. Indian*	*Cult. Kenyan*	*Cult. Brutte*
caffeic acid	10.3	10.1	9.8	10.5	10.2	10.6	10.3	10.2	10.1	9.9	10.0
ferulic acid	3.8	3.7	3.6	3.9	3.8	3.5	4.1	3.9	3.2	3.7	4.2
sinapic acid	4.7	4.4	4.2	4.3	4.9	5.2	5.1	5.0	4.9	4.8	4.6
gallic acid	1.4	1.9	2.0	2.3	2.0	1.8	1.9	1.8	2.0	2.1	2.4
protocatechuic	0.33	0.81	0.41	0.78	0.77	0.81	0.43	0.67	0.89	0.81	0.79
p-hydr.oxy benzoic	0.61	0.99	0.65	0.70	0.81	0.65	0.82	0.61	0.77	0.67	0.87
p-coumaric	14.1	14.2	13.9	13.8	14.4	14.1	13.8	14.3	14.0	14.1	13.8
Phenol	3.6	3.7	3.6	3.4	3.5	3.9	4.0	3.7	4.0	3.8	4.0
o-coumaric	4.2	4.5	4.6	4.4	4.1	4.0	5.0	4.5	4.1	4.9	4.4
Chlorogenic acid	13.1	13.4	14.1	13.7	13.8	13.3	14.0	14.7	14.0	14.1	13.8
Unknown	9.9	10.0	10.3	10.9	10.4	9.9	10.9	9.7	10.6	9.1	10.8
Unknown	7.5	7.8	6.9	7.9	7.3	7.1	7.8	8.9	9.1	9.9	9.7
Unknown	8.9	9.2	10.9	9.2	10.1	10.2	10.1	9.1	10.2	9.8	10.8
Unknown	10.1	10.3	9.9	10.4	10.0	10.1	10.6	9.9	10.0	9.8	10.1

Culti = Cultivar

II - Seed Protein data

SDS-PAGE seed storage proteins analysis was conducted to elucidation the taxonomic relationships between the cultivars 20 different bands ranged between Rf of 0.25 - 0.83. The maximum number of 18 bands were detected in cv. *Colombian* while the minimum numbers of 15 bands are detected in cultivars of *Chinese, Habsh, Ethiobian Harar* and *Brazilian*. Also, all cultivars are shared in 14 bands (Table 5, bands, no. 4, 5, 6, 9, 10, 11, 13, 15, 17, 19, 21, 22, 23 & 24).

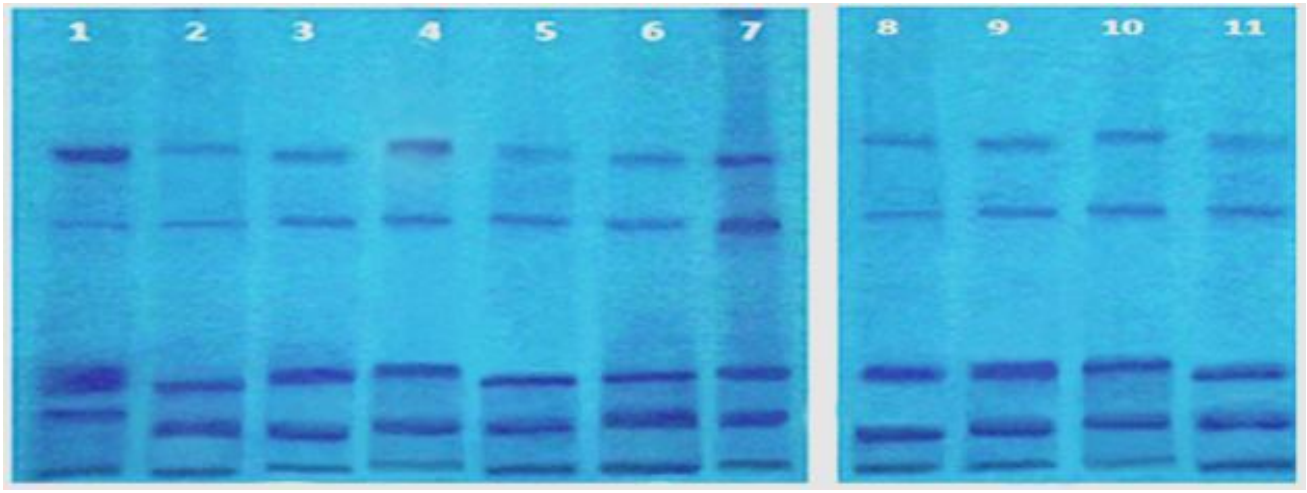

Fig. 2: Seed protein bands of *Coffea arabica cultivars; 1- jazani, 2- Yemeni, 3-Chinese, 4- Colombian, 5- Habshi, 6- Yafi, 7- Ethiobian Harar, 8- Brazilian, 9- Indian, 10- Kenyan, 11- Brutte*

On the other hand, band no. 1 is distinct to cv. *Yemeni*, *Colombian* and *Kenyan*. Band no. 10 is distinct in cultivar *Brazilian* and *Ethiobian Harar*. no. 12 found in all studied cultivars except the two cultivars of *Brazilian* and *Ethiobian Harar*. *Jazani, Yemeni, Chinese* and *Yafi Chinese* are shared in Band no. 16.

Table 5: The migration distance of protein bands between the cultivars

Cultivars / RF	*Cult.* jazani	*Cult.* Yemeni	*Cult.* Chinese	*Cult.* Colomb.	*Cult.* Habshi	*Cult.* Yafi	*Cult.* Ethiobian Harar	*Cult.* Brazilian	*Cult.* Indian	*Cult.* Kenyan	*Cult.* Brutte
0.**25**	--	2.5	--	1.9	--		--	--	--	2.2	
0.**33**	3.0	3,7	7.26	5.9	16.9	7.0	4,5	5.9	3-7	3.7	6.9
0.**34**	2.7	2.6	9.21	7.0	5,9	3.11	5.8	5.31	4.6	11.14	1.23
0.**37**	3.0	3,6	7.4	6.1	6.5	5.8	4.2	5.3	5.2	4.9	3.7
0.**39**	--	--	--	3.8	--	--	--	--	--	--	
0.**40**	3.0	3,7	7.5	5.9	16.9	7.0	4,4	5.9	4-8	3,7	6.9
0.**47**	2.7	2.6	5.4	8.3	6.2	5.9	5.8	2.9	3.12	7.9	8.7
0.**55**	3.0	7.7	7.4	6.9	6.15	5.9	5.8	6.9	5.9	6.9	6.7
0.**58**	6.4	3,7	7.26	7.9	16.9	7.2	5.5	5.9	6.9	6.2	6.9
0.**59**	--	--	--	--	--	--	3.7	2.6	--	--	--
0.**66**	3.0	3,7	7. 6	5.9	16.9	7.0	4.5	5.9	6.4	3,7	8.9
0.**67**	2.6	2.3	2.7	2.4	9.7	6.6	--	--	2.7	2.6	2.7
0.**68**	3.0	3.7	7.9	6.9	6.5	5.9	4.8	5.5	5.9	6.9	3,7
0.**71**	--	--	--	---	--	--	--	--	11.6	6.9	4.32
0.**75**	12.3	4.3	9.6	3,9	7.9	2.9	4,6	5.9	6,6	3,4	5,8
0.79	7.9	5.9	--	6.6	--	7.9	--	--	--	--	--
0.**77**	3.4	3,7	3.6	4.10	6.6	2. 1	5.8	4.9	4.50	6.93	2.5
0.**81**	5. 2	9.6	7.26	5.9	16.9	7.0	4,5	2.9	6.9	8.0	4.9
0.87	2.7	2.6	12.3	15.9	6.9	7.5	8,5	2.7	6.9	8.6	4.0
0.**83**	3.0	3,7	7.4	6.9	6.5	5.9	4.8	5.9	5.9	6.9	3.7
Total	16	17	15	18	15	16	15	15	16	17	16

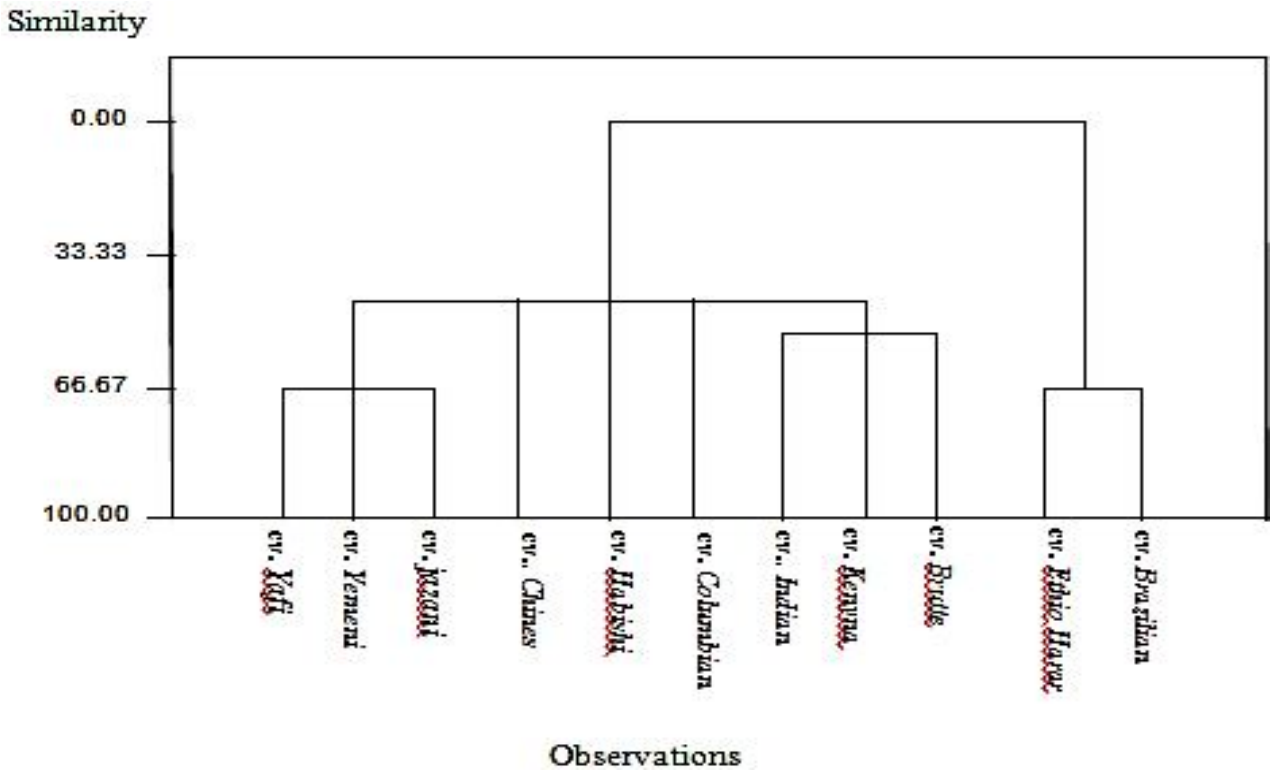

Fig 3: The relationships among the *Coffea arabica* cultivars based on the biochemical and molecular data.

In Figure 3, two groups are recognized, the first included cv. *jazani*, cv. *Yemeni*, cv, *Chinese*, cv. *Colombian*, cv. *Habshi*, cv. *Yafi*, cv. *Indian*, cv. *Kenya* and cultivar *Brutte*. The second group included the two cultivars of *Ethiobian Harar* and *Brazilian*. Three subgroups are recognized in the first group, the first subgroup comprised cultivar *Yafi*, cultivar *Yameni* and *Jazani*; the second included cultivars of *Chinese, Habshi* and *Colombian* meanwhile the third subgroup involved the three cultivars of *Indian*, *Kenyan* and *Brutte*. The second group included the two cultivars of *Ethiobian Harar* and *Brazilian*. From the HPLC results, *p*-coumaric, caffeic and chlorogenic acids are the main components of the phenolic fraction of coffee beans in the studied cultivars. The highest levels of phenolic acids, especially chlorogenic acids and related compounds, in the cultivars of *Brazilian*, *Ethiobian Harar, Yemeni* and *Indian* coffee increase their physiological importance, as well as their contribution to flavor and aroma of coffee beverage. Chlorogenic acids (CGA) are the main phenolic compounds in coffee, being esters of trans-cinnamic acids, such as caffeic, ferulic and p-coumaric acids which in accordance with results of Qais *et al*., 2013) [11]. These highest concentrations of these constituents play an important role in the formation of coffee flavor and have a marked influence in determining coffee quality; while the flavone compounds of protocatechuic and *p*-hydroxy benzoic are the lowest acid concentration. This variation of different phenolic acids is due to different factors such as environmental conditions, genetic, species and variety, physiological factors and the degree of maturation. This conclusion was in accordance with results of ower biochemical data. However, *cv. brazilian* and *cv. Etiobian Harar* were quite distinct from the others by their a distinct values of *p*-coumaric, caffeic and chlorogenic acids which in turn give a good flavor which is in accordance with the work of Monteiro and Farah (2012) [10]. In addition three alkaloid spots are noticed by TLC in *cv. Brazilian.* Also, three alkaloid spots appeared in the three cultivars of *Yafi*, *Ethiobian Harar* and *Brazilian*. On the other hand, there are a significant correlation between the three cultivars of *Indian, Kenyan, Brutte* from point of view phytochemical data. Also, these three cultivars resemble in the total chemical constituents. It is obvious that, there are differences appeared between the two cultivars of *Brazilian* and *Ethiopian Harar* by SDS-PAGE of seed proteins than the remainder cultivars. Such result was not agreed with the results of Sandra *et al*., 2001 [19]. These two cultivars are characterized with presence of a distinctive protein band no. 10. Also, Columbian cultivar has a distinct protein band no. 5 and high caffeic acid content (10.5). In conclusion, the data obtained from both chemical constituents and seed protein studies were useful as good taxonomic purposes in the coffee cultivars.

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