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Resveratrol content and its losses upon processing in select peanut accessions

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Abstract
Peanuts, a healthy snack across the globe, are considered good dietary source of resveratrol. Twenty diverse peanut accessions were studied for resveratrol content and its losses during processing – roasting, boiling, and combination of roasting and peeling. Resveratrol was quantified using LC-MS/MS. Based on average silhouette score and hierarchical clustering, accessions were grouped into three clusters. Resveratrol in raw peanut kernels ranged from 58 to 619 µg/kg. All processing methods resulted in resveratrol loss. Roasting, boiling, peeling after roasting resulted in 6 – 88%, 27 – 94%, 46 – 100% loss respectively. Of the treatments studied, ICGV-00440 showed minimum loss of resveratrol at 6% during roasting while highest loss of 100% was observed in ICGV-SM 86068 when it was subjected to roasting followed by peeling. ICGV 06040 and ICGV-13189 were found to be best accessions for roasting and boiling, respectively. Overall, roasting resulted in minimum loss of resveratrol compared to other studied processing methods.

Keywords: resveratrol, stilbenoid polyphenol, peanut nutrition, processing losses

1. Introduction
Peanut or groundnut (*Arachis hypogaea* L.) is a self-pollinated legume crop and is an allotetraploid (2n =2x =40) with "AA" and "BB" genomes of total 2.7 Gb genome size [1, 2]. It is mostly grown in semi-arid tropic regions in over 100 countries of Asia, Africa and Americas occupying over 28.5 million hectares of cultivated area globally with 45.95 million tons of produce being harvested during 2018 [3]. Peanut seeds contain valuable rich source of energy component like oil (48-50%) protein (25-28 %) and carbohydrates (18%). In addition, they contain many other important nutritional components such as iron, zinc, calcium, magnesium, phosphorus, folate, niacin, vitamin E, potassium and fatty acids. Peanut kernels are most important part of the plant as it is used for the oil extraction, cooking, eaten as boiling/ roasting and fresh, used in making for peanut butter, peanut cake, chikki, and many other confectionary items [4]. It is also known as poor man's almond because of its high nutritious value and in fight against malnutrition.

Peanut contains dietary source of bioactive compounds such as polyphenols, isoflavones, flavonoids and stilbenes. These bioactive compounds are highly beneficial to the body and has functioning as an antioxidant [5]. Peanut contains many antioxidants such as p- coumaric acid, resveratrol and vitamin E. The phytoalexin, stillbenoid resveratrol (3, 5, 4′-trihydroxy-trans-stilbene), was first isolated by Takaoka from the roots of white hellebore in 1940 [6]. It is mostly found in peanut, peanut butter, grapes and red wines [7]. The *trans*- resveratrol possessing two phenolic rings linked to each other by a styrene double bond with a molecular weight of 228 (Figure 1). The *trans*-form is more stable form [8]. So, the *trans*-form is dominant in terms of its prevalence and is high in bioactive effects. Resveratrol is biosynthesized from the p-coumaric acid with the help of resveratrol synthase [6]. It is a potent inhibitor of the reactive oxygen species, hence has effective antioxidant properties associated with cardiovascular protective effect, delaying aging, neurodegenerative disease such as Parkinson's and Alzheimer's disease, reducing cancer, phytoestrogen activity, reducing pain and hyper glycemia.

As per the literature most of the researches focused their work on determination of resveratrol content in raw or roasted peanuts and the work also limited to particular region [9, 10] or specific cultivars [11] or specific method of germination [12, 13]. But the present study was undertaken to quantify the resveratrol content in genetically diverse accessions which are being cultivated in different parts of the world. The amount of resveratrol was analyzed in raw peanuts followed by determining its loss after roasting, boiling, and peeling subsequent to roasting. Hyphenated technique i.e., chromatography coupled with mass spectrometry (LC-MS/MS) was adopted for the analysis of resveratrol in peanut. In this manuscript, the word resveratrol refers to *trans*-resveratrol only.

2. Materials and Methods
Twenty peanut accessions, as described in Table 1, were used for studying the total resveratrol content in raw peanuts and its losses upon processing. The selected accessions represent a wider genetic diversity as they are grown in many countries across Asia and Africa including India, Nepal, Pakistan, Philippines, Sri Lanka, Ghana, Malawi, Mozambique, South Africa, Uganda, Vietnam, Zambia, Zimbabwe. These accessions were grown in ICRISAT's research plots during post-rainy 2018-19 and stored at 4 degree centigrade for 2 months prior to analysis. Methodology followed is diagrammatically represented in Figure 2. Certified reference material of resveratrol (catalogue number: R150000) used for preparation of

calibration standards and quality control checks was purchased from Toronto Research Chemicals, Canada.

2.1 Sample preparation

2.1.1 Roasting

About 50 g of peanut kernels were taken in a clean stainless-steel container and kept in hot air oven maintained at 110ºC for two hours. It was ensured that the kernels won't get burnt by occasionally stirring with a clean wooden spatula. The roasted kernels were cooled to room temperature prior to proceeding with extraction procedure as described in section 2.2

2.1.2 Boiling

About 50 g of peanut kernels were taken in a 500-ml clean corning glass beaker to which distilled water was added. The beaker containing peanut kernels was covered with a watch glass and kept on a hotplate maintained at 100ºC for three hours. During boiling, water level was maintained just above the peanut kernels by adding hot water which was also maintained at the same temperature of boiling kernels. Upon completion of boiling, water was drained from the beaker and kernels were spread on blotting paper to remove excess water. Dried peanut kernels were subjected to extraction procedure as described in section 2.2

2.1.3 Peeling subsequent to roasting

Roasted peanut kernels, as described in section 2.1.1, were subjected to peeling by hand. Peeled kernels were separated from the skin and were extracted as described in section 2.2

2.2 Extraction

For sample extraction, the method described by Lee et.al. (2004) [9] with few modifications was followed. Peanut samples were finely ground using a blender. From the homogenized sample, about 5 g was accurately weighed in a 50-ml polypropylene centrifuge tube to which 25 ml of extraction solvent (acetonitrile and water in 8:2 ratio, v/v) was added. The mixture was homogenized with digital ultra turrax (Make: Ika, Model: T25) at 10000 RPM for three minutes and incubated for 30 minutes at 70ºC in water bath. Sample was cooled to room temperature and centrifuged at 4000 RPM for five minutes. From the supernatant, 1-ml was taken for clean-up with C18 50 mg by using dispersive solid phase extraction technique to remove potential interferences. The extract was vortexed and centrifuged at 15000 RPM for five minutes and filtered with 0.22 µm nylon syringe filter (Catalogue number CH2225-NN purchased from Thermo Scientific). From the filtrate, 0.5 ml was taken and diluted to 1-ml using extraction solvent. Such obtained extract was injected into LC-MS/MS for resveratrol determination.

2.3 LC-MS/MS analysis

Extracts were analyzed for resveratrol using LC-MS/MS (Make: Waters Corporation, USA; Model: TQS Micro) due to its higher sensitivity and selectivity compared to other methods of analysis including HPLC. Column used for resveratrol determination was BEH C18, 130Å, 1.7 µm, 2.1 mm x 50 mm purchased from Waters Corporation, USA. Two mobile phases – A) 98% Water + 2% Acetonitrile with 0.1% Formic acid; B) 98% Acetonitrile + 2% Water with 0.1% Formic acid, were used as per the gradient given in Table 2. Addition of formic acid was reported [14] to improve the ionization efficiency in positive ESI mode. The injection volume was 5µl with a run time of six minutes.

The mass spectrophotometer was operated in positive electrospray ionization (ESI) mode for resveratrol determination. The multiple reaction monitoring (MRM) mode was used with a dwell time of 50ms including three transitions for each analysis. Employed mass spectrometric parameters are given in Table 3.

Aqueous 6-point calibration, excluding calibration blank, was performed in the range of 1 – 100 ppb using resveratrol certified reference material. Method validation and calibration acceptance criteria as given in SANTE guidelines [15] was followed. For the calibration to be acceptable, deviation of the back calculated concentration should be within ±20% of the true concentration. Calibration plot is shown in Figure 3. Acquired data was quantified using the software, Mass Lynx 4.1 version.

3. Results and Discussion

Resveratrol content showed a wide variation amongst the 20 accessions studied. Its content in raw peanuts ranged from 58 to 619 µg/kg (Table 4). ICGV 06040 had the highest content of resveratrol in raw peanuts as well as showed maximum content after roasting and peeling subsequent to roasting. However, ICGV 13189 showed highest content (167 µg/kg) of resveratrol upon boiling.

Using R programming language [16], all the accessions were subjected to Average Silhouette score to determine the optimum number of clusters for grouping the studied accessions. Results indicate that the optimum number of clusters for grouping studied accessions is three (Figure 4). Subsequently, hierarchical clustering technique was used for grouping the studied accessions using R programming language. Obtained cluster dendrogram is shown in Figure 5. From the dendrogram it can be clearly seen that accession, ICGV 00440, is dissimilar than all other accessions studied. Based on the data, clusters have been named as – Low, Medium, and High whose mean resveratrol loss of each treatment and average loss across treatments are given in Table 5.

However, since the accessions are known to be diverse with respect to their genetic background, grouping was further performed into five clusters as shown in Figure 6. It is interesting to note that the accession ICGV 00440, which was found to be dissimilar amongst all studied accession when categorized into three clusters, still remained in a separate cluster indicating its significant dissimilarity amongst studied accessions. However, accession ICGV 89322 which was part of a cluster along with ICGV 02266, ICGV 86015, and ICGV 93437 when grouped into three clusters, fell into a separate cluster on its own indicating slight dissimilarity.

All the accessions studied showed a decrease in the content of resveratrol upon processing compared to their content in raw peanut kernels. Sanders et.al. (2000) [17] obtained similar results in Virginia and Spanish peanuts which showed a decrease in *trans*-resveratrol content in roasted peanuts compared to raw peanut kernels. However, contrasting observations were made by Rudolf (2003) [18] where the *trans*-resveratrol content increased in ultrasound-stressed peanuts. This might possibly due to the application of abiotic stress which can result in increased *trans*-resveratrol content. Sales and Anna (2014) [6] reported that stilbenes including resveratrol accumulate in plants due to

biotic and abiotic stresses, which activate stilbene synthase, an enzyme required for synthesis of resveratrol. Biotic stresses included invasion of biological agents while abiotic stresses included physical, mechanical, or chemical agents such as wounding, exposure to UV light, ultrasound, treatment with metallic salts etc. As expected, when roasted kernels were peeled, they showed lower content of resveratrol than roasted kernels indicating presence of resveratrol in peanut kernel skins. Reduction of resveratrol in such peeled kernels ranged from 46 to 100% while roasting alone resulted in loss of 6 – 88% when compared to raw peanut kernels. Further analysis of the data indicates, peeling of kernels subsequent to roasting showed a loss of 5 – 100% in comparison to roasted kernel's resveratrol content. These results highlight the importance of retaining kernel skin during consumption for greater availability of resveratrol. In general, roasting retained higher amounts of resveratrol among the three processing methods studied (Figure 7). Like roasting, boiling has also resulted in loss of resveratrol content ranging from 27 – 94% in comparison to raw peanut kernels. ICGV 89322 was found to lose least amount (27%) upon boiling while ICGV 90320 showed maximum loss at 94%. Except for four accessions (ICGV 91114, ICGV SM 86068, ICGV SM 90704, and ICGV 89322), in all other accessions boiling had resulted in higher losses compared to roasting. But the losses were higher upon roasting followed by peeling in comparison to boiling for all accessions except ICGV 06040, ICGV 86590, ICGV 00298, ICGV 00440, and ICGV 90320 (Figure 6). However, Chukwumah et.al. (2007) [19] reported an increase in the content of *trans*-resveratrol in commercial peanuts upon boiling. The increase in content was attributed to potential migration of compound of interest from the hull. However, resveratrol content in peanut shells was not reported. It will be interesting to quantify the resveratrol content, if any, in peanut shell. In our study, since peanut kernels were subjected to boiling after dehulling, such migration from hull is not possible. Losses during boiling may possibly be due to hydrolysis of resveratrol. Ahmad et.al. (2018) [20] also reported loss in resveratrol due to roasting and boiling in Melinjo (*Gnetum gnemon* L.) seeds.

4. Tables and Figures

Table 1: Details of diverse peanut accessions phenotyped for resveratrol after roasting, boiling, and combination of roasting and peeling

Sr. No.	Accession name	Species	Sub species	Botanical type	Agronomic type	Biological Status	Origin	Region/Comment
1	ICGV 06420	*A. hypogaea*	*hypogaea*	*hypogaea*	Virginia bunch	Advanced/ Improved	ICRISAT	High Oil
2	ICGV 13189	*A. hypogaea*	*hypogaea*	*hypogaea*	Spanish bunch	Advanced/ Improved	ICRISAT	Early + FDR
3	ICGV 15426	*A. hypogaea*	*fastigiata*	*fastigiata*	Spanish bunch	Advanced/ Improved	ICRISAT	Fresh seed dormancy
4	ICGV 06146	*A. hypogaea*	*fastigiata*	*Vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Disease resistant_ Tamil Nadu
5	ICGV 00350	*A. hypogaea*	INA	INA	INA	Advanced/ Improved	ICRISAT	Drought tolerant
6	ICGV 06040	*A. hypogaea*	*fastigiata*	*fastigiata*	Spanish bunch	Advanced/ Improved	ICRISAT	Fe and Zn
7	ICGV 02266	*A. hypogaea*	*fastigiata*	INA	INA	Advanced/ Improved	ICRISAT	Dual purpose
8	ICGV 91114	*A. hypogaea*	*fastigiata*	*Vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Short duration_ AP
9	ICGV 86015	*A. hypogaea*	*fastigiata*	*Vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Short duration_ Nepal, Pakistan, Sri Lanka, Vietnam
10	ICGV 93437	*A. hypogaea*	*fastigiata*	*Vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Short duration_ Zimbabwe, South Africa, Zambia
11	ICGV 87141	*A. hypogaea*	*hypogaea*	*virginia*	Virginia bunch	Advanced/ Improved	ICRISAT	High yield_ South Maharashtra, AP, Kerala, Karnataka, Tamil Nadu
12	ICGV SM 86068	*A. hypogaea*	*fastigiata*	*vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	High yield _ Zimbabwe
13	ICGV SM 90704	*A. hypogaea*	*hypogaea*	*hypogaea*	Virginia bunch	Advanced/ Improved	ICRISAT	High yield_ Uganda, Malawi, Zambia, Mozambique
14	ICGV 89322	*A. hypogaea*	INA	INA	INA	Advanced/ Improved	ICRISAT	Confectionary_ Uganda, Malawi, Zambia
15	ICGV 86590	*A. hypogaea*	*fastigiata*	*fastigiata*	Valencia Bunch	Advanced/ Improved	ICRISAT	Disease resistant_ Maharashtra, Kerala, Karnataka, Tamil Nadu
16	ICGV 00298	*A. hypogaea*	INA	INA	INA	Advanced/ Improved	ICRISAT	New Season: UP
17	ICGV 00440	*A. hypogaea*	*hypogaea*	*hypogaea*	*Virginia bunch*	Advanced/ Improved	ICRISAT	Confectionary: Malika_India
18	ICGV 86564	*A. hypogaea*	*hypogaea*	*hypogaea*	*Virginia bunch*	Advanced/ Improved	ICRISAT	Confectionary: Asha_ Sri Lanka, Philippines

19	ICGV 90320	*A. hypogaea*	*fastigiata*	*vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Confectionary: NAMNAMA 1_ Philippines
20	ICGV 98412	*A. hypogaea*	*fastigiata*	*Vulgaris*	Spanish bunch	Advanced/ Improved	ICRISAT	Confectionary: Obooshi_ Ghana
INA: Information Not Available								

Table 2. Details of diverse peanut accessions phenotyped for resveratrol After roasting, boiling, and combination of roasting and peeling

Table 2: Mobile phase gradient profile for resveratrol determination

Time	Flow Rate (ml/min)	% A	% B
0.00	0.30	90	10
2.00	0.30	20	80
4.00	0.30	10	90
4.10	0.30	90	10
6.00	0.30	90	10

Table 3: Mass spectrometric parameters for estimation of resveratrol content

Source Parameter			Value		
Polarity			+ve		
Capillary voltage			3.0 KV		
Desolvation gas flow			1000 L/hr		
Source temperature			150ºC		
Cone gas flow			50 L/hr		
Desolvation temperature			500ºC		
Parent Ion (Q1)	Product Ion (Q3)	Dwell time (ms)	Quantifier/ Qualifier	Cone voltage (V)	Collision energy (V)
229	107	50	Quantifier	25	22
229	135	50	Qualifier	18	12
229	91	50	Qualifier*	34	21

* Second qualifier, where required

Table 4: Resveratrol content and its losses upon processing in Select Peanut accessions

Accession	Accession Code	Resveratrol Content (µg/kg)				% loss due to		
		Raw	Roasted	Boiled	Roasted & Peeled	Roasting	Boiling	Roasting & Peeling
ICGV 06420	1	233	127	56	52	45	76	78
ICGV 13189	2	488	276	167	138	43	66	72
ICGV 15426	3	191	96	41	5	50	79	98
ICGV 06146	4	199	81	37	23	59	81	89
ICGV 00350	5	329	84	72	24	74	78	93
ICGV 06040	6	619	324	79	189	48	87	69
ICGV 02266	7	205	162	91	36	21	56	82
ICGV 91114	8	300	91	98	35	70	67	88
ICGV 86015	9	128	85	75	15	34	41	89
ICGV 93437	10	92	72	33	15	21	64	84
ICGV 87141	11	248	94	36	20	62	86	92
ICGV SM 86068	12	76	13	19	0	83	75	100
ICGV SM 90704	13	168	35	47	33	79	72	80
ICGV 89322	14	143	61	104	37	58	27	74
ICGV 86590	15	58	19	11	12	68	81	80
ICGV 00298	16	82	34	17	28	58	79	66
ICGV 00440	17	123	116	51	66	6	59	46
ICGV 86564	18	397	47	42	37	88	89	91
ICGV 90320	19	145	63	8	15	57	94	90
ICGV 98412	20	197	69	53	9	65	73	95

All results reported on dry weight basis.

Table 5: Clustering of accessions based on resveratrol content and cluster mean resveratrol losses due to various processing methods

Cluster Name	No. of accessions in the cluster	% Resveratrol loss due to			Average loss (%)
		Roasting	Boiling	Roasting & Peeling	
Low	1	6	59	46	37
Medium	4	34	47	82	54
High	15	63	79	85	76

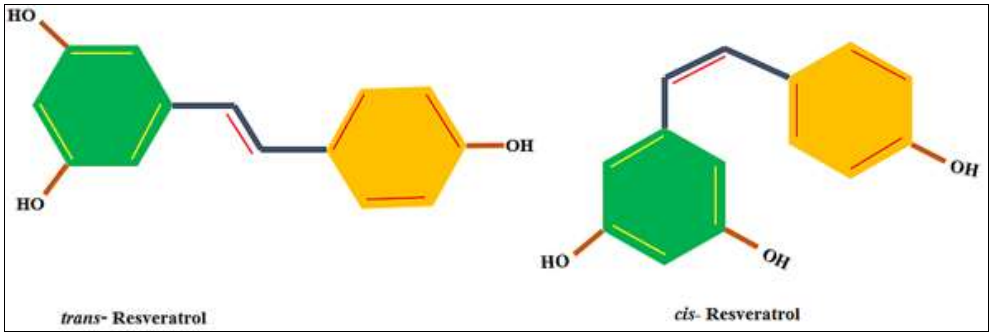

Fig 1: Chemical structure of *cis*- and *trans* isoforms of resveratrol

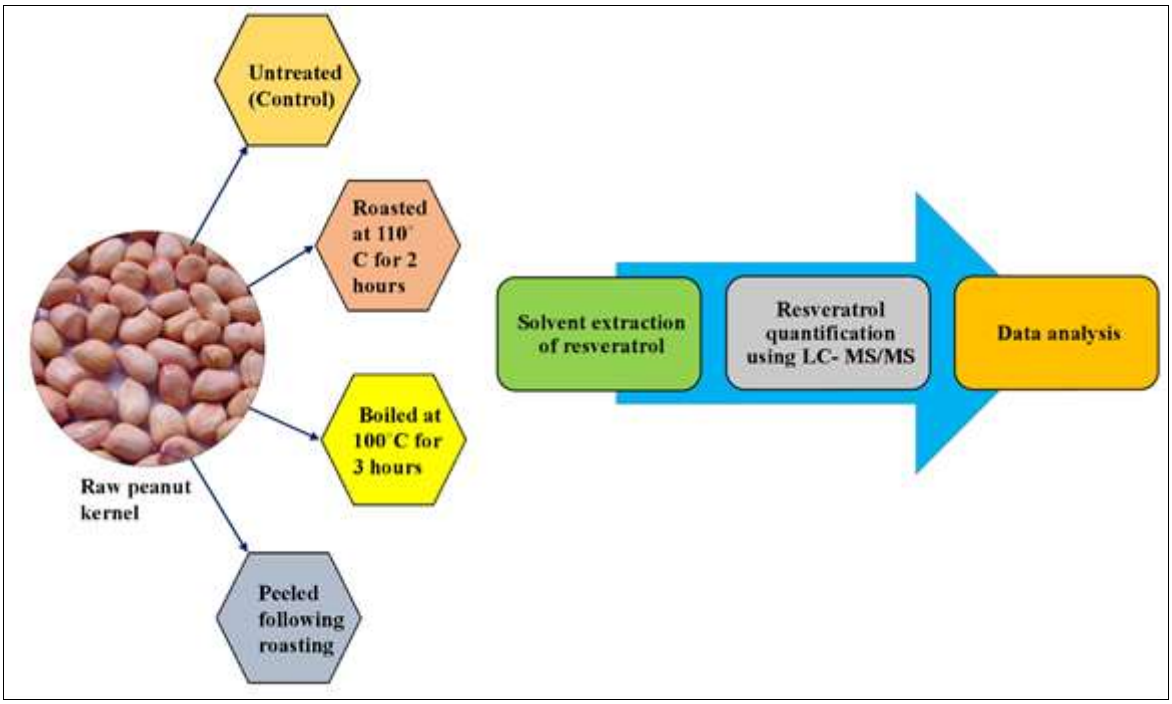

Fig 2: Diagrammatic representation of methodology

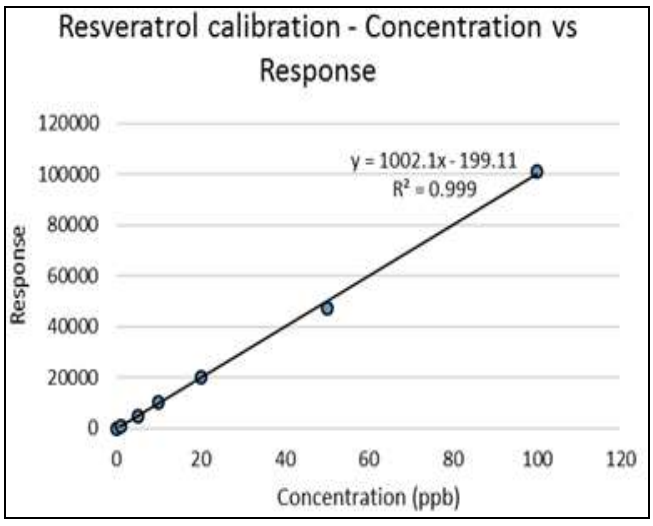

Fig 3: Linear calibration plot of resveratrol

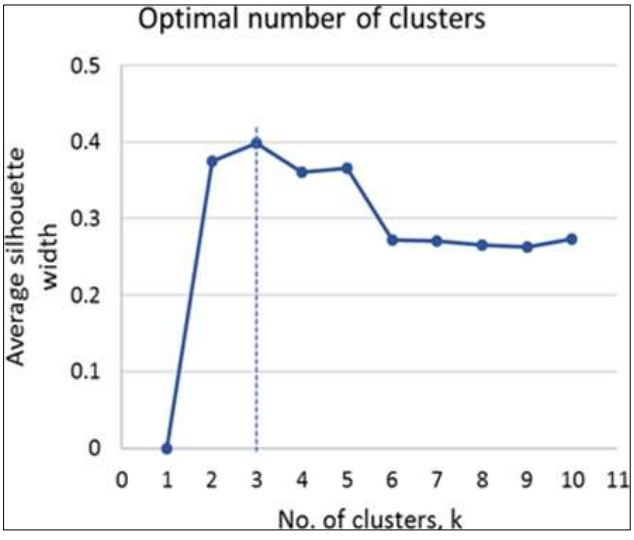

Fig 4: Optimal number of clusters based on average silhouette method

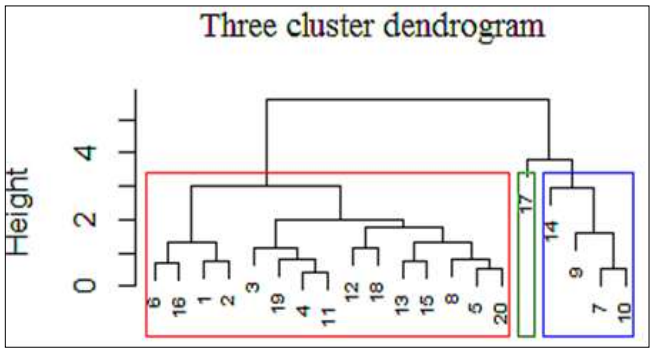

Fig 5: Three cluster dendrogram based on resveratrol content (Accessions have been indicated as per the accession code assigned in Table 4)

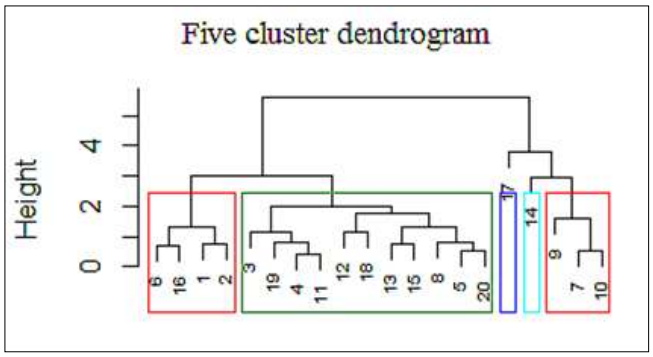

Fig 6: Five cluster dendrogram based on resveratrol content (Accessions have been indicated as per the accession code assigned in Table 4)

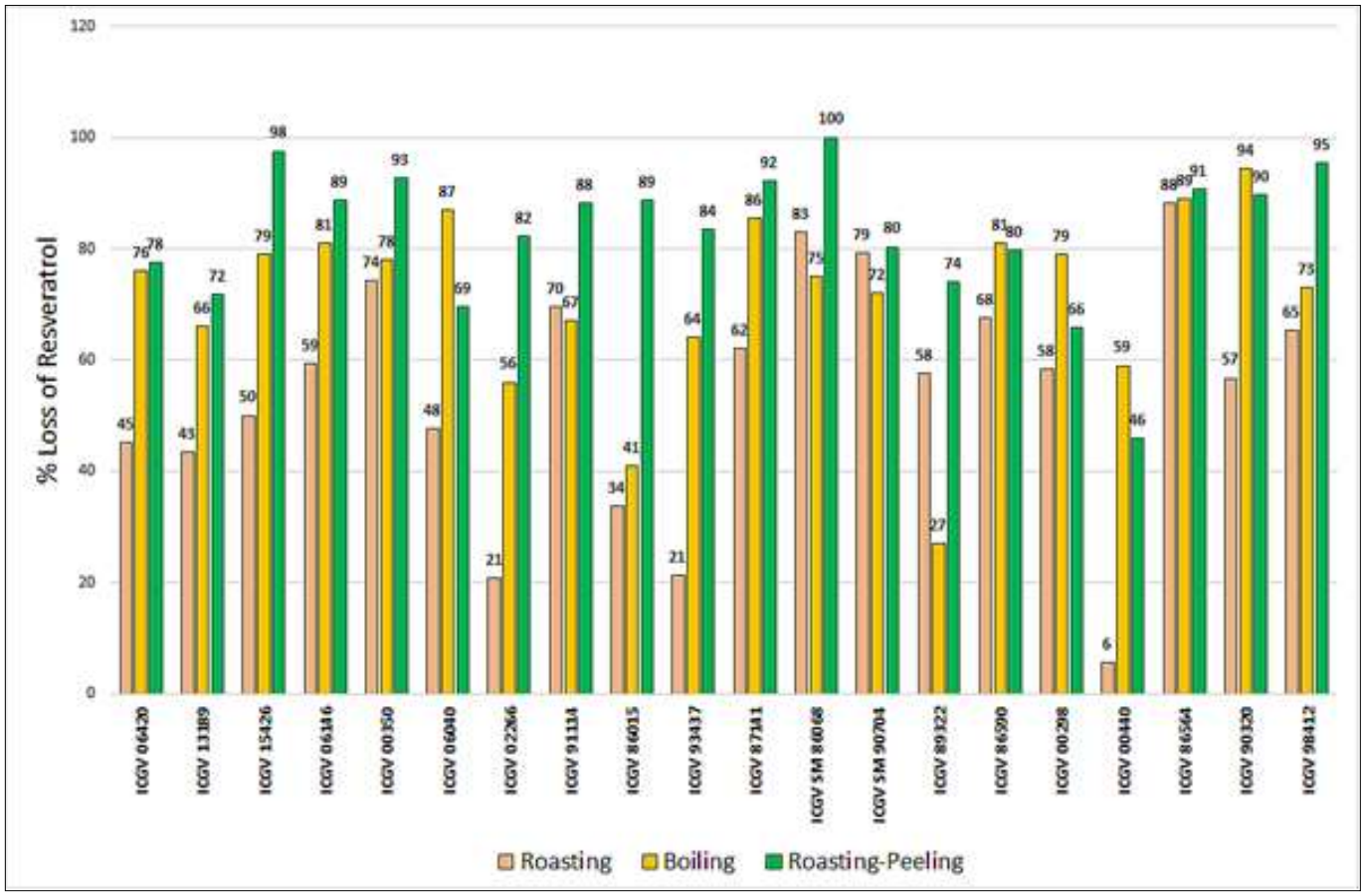

Fig 7: Percent losses in Resveratrol content due to processing in select peanut accessions

5. Conclusions

All three types of treatments – boiling, roasting, and peeling subsequent to roasting, resulted in significant loss of resveratrol in all the accessions studied. Of the treatments studied, ICGV 00440 showed minimum loss of resveratrol at 6% during roasting while highest loss of 100% was observed in ICGV SM 86068 when it was subjected to roasting followed by peeling. ICGV 06040 was found to be the best accession for roasting while ICGV 13189 was best for boiling as they were found to retain maximum amount of resveratrol after treatment. Overall, roasting has resulted in maximum retention of resveratrol compared to other studied processing methods. While roasting showed a loss of 6 – 88%, boiling resulted in 27 – 94% loss. Maximum loss, 46 – 100%, was observed when peanuts were peeled subsequent to roasting. It indicates that skin of peanut kernels contains significant amount of resveratrol. Based on average silhouette score and hierarchical clustering, accessions were grouped into three clusters. Accession ICGV 00440 seems to be significantly dissimilar from other accessions. It was further confirmed when the accessions were grouped into five clusters. Further studies must be undertaken to determine the effect of duration and temperature of different treatments to establish ideal combination for minimizing resveratrol losses so as to derive maximum health benefits.

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CGIAR.

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