# CYTOGENETICAL EFFECT OF SOME POLYPHENOL COMPOUNDS SEPARATED FROM INDUSTRIAL BY-PRODUCTS ON MAIZE (*Zea mays* L.) PLANTS

CORNELIU TANASE,[*] CSILLA IULIANA BARA[**] and VALENTIN I. POPA[***]

[*]*University of Medicine and Pharmacy of Tirgu Mureș, Faculty of Pharmacy, Pharmaceutical Botany Department, 38, Gh. Marinescu Str., 540139, Tirgu Mures, Romania*
[**]*"Alexandru Ioan Cuza" University of Iasi, Faculty of Biology, Vegetal Biology Department, 11, Carol I Blvd., 700506 Iasi, Romania*
[***]*"Gheorghe Asachi" Technical University of Iasi, Faculty of Chemical Engineering and Environmental Protection, 73, Prof. Dr. Doc. D. Mangeron Str., 700050 Iasi, Romania*
✉ *Corresponding author: Corneliu Tanase, tanase.corneliu@yahoo.com*

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Plant growth and development are influenced by various exogenous and endogenous factors. In this context, a group of substances of particular interest are polyphenols. Considering the information presented in the literature data and the results obtained by our working group to date, our objective has been to study the influence of some polyphenolic extracts on the mitotic division. The investigated material comes from the seeds of maize, untreated (control) and treated with spruce bark polyphenolic extract (SBPE), hemp shives polyphenolic extract (HSPE) in different concentration. It was found that using SBPE or HSPE with lower concentration, the frequency of cells in mitosis was higher than in the control. At higher concentrations of the extracts, there were a higher number of cells with chromosomal aberrations. In the treatment variants, significant proportions had an ana-telophase (A-T) with bridges, delayed chromosomes and complex aberrations such as: A-T + bridges + late and/or expelled chromosomes, A-T multipolar + bridges. By using the RAPD technique, it was found that the effect generated by the SBPE and HSPE in high concentrations was the inactivation of DNA (deoxyribonucleic acid).

***Keywords***: mitotic division, polyphenols, spruce bark, hemp shives, maize

## INTRODUCTION

The choice of maize (*Zea mays* L.) as "target species" is justified by the fact that this plant is one of the main sources of carbohydrates, minerals and nitrogenous substances as part of the daily diet of the inhabitants of many countries, both in Europe and worldwide.[1] The crops of maize found in Romania favourable natural conditions to reach high yields and quality. Maize is used as raw material for many industries such as the food and pharmaceutical industries and for animal feed.

Currently, about 75% of the world population depends on herbal extracts and modern pharmacopoeia still contains at least 25% of drugs derived from plants and a series of synthetic analogues are synthesized using as prototype a series of compounds isolated from plants.[2] Numerous researches have been conducted to identify compounds that may protect against DNA damage and the consequences resulting therefore. Throughout the world, efforts are continuously made to explore the rich biodiversity of medicinal and food plants to find the most efficient phyto-antimutagens. These bioactive compounds belong to different chemical groups, such as phenol, allyl sulfide compounds, tannins, anthocyanins, tioglicozide, flavonoids, phytosterols, protease inhibitors, phytoestrogens. Many of these substances have antimutagenic and anticarcinogenic properties.[3,4]

In recent years, there has been a wide interest in finding phytochemicals from natural sources that could replace synthetic antioxidants, such as butylated hydroxytoluene or hydroxyanisole, which are commonly used in the food industry, because of their toxicity.[5] Compounds such as

phenolics, flavonoids, terpenoids, flavonols, tannins and proanthocyanidins, are found in most plant products.[6] Polyphenols are a large group of natural compounds, which possess a wide spectrum of biological activity.[7] Plant phenols exhibit significant antioxidant, antitumoral, antiviral, anti-inflamatory, antibiotic properties and allelopathic activities.[8,9,10] Through their characteristic biological activity, natural polyphenols are essential compounds in the stimulation of plant growth and development.[11,12] The effect of natural polyphenols in plant growth is correlated with the concentration of these compounds. In previous studies, it was established that spruce bark polyphenolic extract had a stimulating effect on the germination processes,[13] plant cultivation,[12] tissue culture[14] in seedling of rape (*Brassica napus* L.), soybean (*Glycine max* L.), sunflower (*Helyantus annuum* L.) and maize (*Zea mays* L.). The influence of polyphenolic compounds on physiological processes (cell division and expansion, membrane permeability, nutrient uptake, water ratio, respiration) was also reported.[15,16] Thus, hemp shives polyphenolic extract, rich in caffeic acid and catechins, has a great influence on rapeseed plantlet growth and development. It was found that the bioactive compounds present in HSPE can modulate the bioaccumulation processes of lead and copper ions in *Brassica napus* L., depending on the heavy metal concentrations.[17]

Starting from the information presented in the literature data and the results obtained by our working group to date, we decided to study the influence of some polyphenolic extracts (SBPE and HSPE) on the mitotic division. Thus, in this paper, we identified the cytogenetic effects of the treatment with natural polyphenolic compounds on maize (*Zea mays* L.) plants.

## EXPERIMENTAL

### Plant materials

Spruce bark was purchased as industrial waste from Alpine LTD Timber Company, Vatra Dornei, Romania. Waste hemp shives, resulted after bast fiber separation, were used in this study. The vegetal material was randomly collected, while the species was identified and authenticated. After drying (at normal temperature and aeration) and grinding, both vegetal materials were extracted using distilled water. 10 g (SB1) and 5 g (SB2) of ground dried spruce bark (*Picea abies* L.) with 0.5-1 mm particle size and 20 g (HS1) and 10 g (HS2) of ground dried hemp shives, with 1-1.5 mm particle size, were extracted using 125 mL distilled water in a water bath, for 45 min at 80 °C during three runs and the extracts were cumulated to a final volume of 500 mL.[18,14] The SBPE and HSPE were characterized from the point of view of dry matter content, total polyphenolic content, total content of tannins, flavonoids, flavonols and antocyanins using selected samples with about the same content of total polyphenols. These results, as well as a detailed description of the extraction method, were published in our previous work.[14,12]

Maize seeds were purchased from a seed distribution company (Unisem Impex SRL, Romania).

### Experimental assay

Germination tests were carried out according to a standard procedure, using a number of 10 Petri dishes for each experimental variant: SB1 (SBPE with 191 mg GAE/L concentration), SB2 (SBPE with 130 mg GAE/L concentration), HS1 (HSPE with 233 mg GAE/L concentration) and HS2 (HSPE with 164 mg GAE/L concentration). Every five maize seeds were placed on a filter paper and those presenting no major damage were carefully selected. For starters, the vegetal material underwent a presterilization process, which consisted in submerging the seeds in absolute ethanol for 10 seconds, following the sterilization in the presence of 10% sodium hypochlorite for 20-30 minutes.[19] The volume of solution added was 10 mL/dish. The Petri dishes thus prepared were incubated in the dark in a thermostat, set at 27 ºC. After a period of three days, the shoot apex (5-10 mm in length) was separated for cytogenetic determinations or amplification using the RAPD method.

### Working protocol for cytogenetic determination

For chromosome coloring, the Feulgen method was used.[20] Hydrolysis was performed with 20% HCl solution at room temperature for 18 minutes, preceded by a washing operation for 5 minutes in a solution of 1N HCl. Coloring was carried out by maintaining the biological material in a 10% solution of carbol-fuxin,[21] in a refrigerator for about 24 hours. Once the roots were obtained, we used the Squash method[22] to emphasise the chromosome aberrations. Also, we took digital camera pictures (Nikon Eclipse 600-Nikon Cool Pix, at 100x immersion objective) of various stages of division for all concentrations and times.[22]

### Determination of DNA variability. Isolation and purification of DNA

This step was accomplished by obtaining the cell sediment, destroying the cell wall by lysozyme, wall lysis and nuclear enzymatic deproteinization followed by precipitation of proteins and nucleic acids. Then, the removal of RNA molecules in the extract, washing the DNA sediment with 70% cold ethanol and the resuspension of the DNA sediment (Table 1) were performed.

**DNA amplification by RAPD method (Random Amplified Polymorphic DNA)**

After DNA isolation and purification follows DNA amplification by the RAPD method.[23,24] DNA amplification was performed in Eppendorf tubes with a volume of 200 µL in a reaction volume of 25 µL. Amplification was conducted in a PX2 thermocycler produced by Thermo Electron Corporation. The reaction mixture (Table 1) containing: 5X Green GoTaq Flexi buffer, 25 mM - $MgCl_2$ solution, dNTPs, and GoTaq DNA polymerase (5 U/µL) that was included in the kit GoTaq Flexi DNA Polymerase (Promega, catalog number M8305). The program used to amplify the DNA: 95 °C - 3 min; 95 °C – 10 s; 45 °C – 10 s; 72 °C - 1 min (45 cycles); 72 °C - 2 min; 4 °C - 10 min.

**Evaluation of RAPD products in agarose gel**

After completion of the amplification cycles, RAPD products were separated by electrophoretic migration in agarose gel. For this stage, the following reagents are required: agarose (1% concentration), TBE (Tris-borate-EDTA) 1X (89 mM, Tris, 89 mM boric acid, 2 mM EDTA); 1X solution of ethidium bromide (0.05 mg-ml); 100 pb molecular weight marker; 6X Loading Dye.

**Statistical analysis**

All the results are expressed as mean ± standard error where $n = 3$. A comparison of the means was performed by the Fisher least significant difference (LSD) test ($p \leq 0.05$) after ANOVA analysis, using program PAST 2.14. Sampling and chemical analyses were performed in triplicate, in order to decrease the experimental errors and to increase the experimental reproducibility.

## RESULTS AND DISCUSSION

### Characteristics of SBPE and HSPE

The characteristics of the spruce bark polyphenolic extract were summarized in another work.[14] The results indicate that the spruce bark polyphenolic extract contains considerable quantities of aromatic compounds, such as vanillic acid (39.4 mg GAE/100 g) and catechine (31 mg GAE/100 g) (Table 2). Total polyphenolic content (TPC) for the use of SBPE is 191 mg GAE/L (SB1) and 130 mg GAE/L (SB2). The SBPE was also characterized in terms of total content of tanins, flavonoids, and flavonols, color intensity and pH.[14]

The HSPE was characterized in terms of dry matter and organic matter, TPC, pH and quantification of components as catechin and phenolic acid (Table 2).[17] The TPC of HSPE is 16.41 mg GAE/100 g and catechine represents 5.21 g GAE/100 g plant material; caffeic acid is present in a higher concentration (9.68 g GAE/100 g plant material) compared with other identified phenolic acids (Table 2).[17] Total polyphenolic content (TPC) for the use of HSPE is 233 mg GAE/L (HS1) and 194 mg GAE/L (HS2).

### Mitotic index and mitotic division phase frequency

The action of mutagenic factors on plant organisms represents an informational message disturbance, a change in the information content itself.[28] To assess the biological effects induced by physical and chemical mutagens in plant bodies, a basic criterion is the assessment of lesions induced at chromosomal level. These lesions will affect individual biochemism, and will be translated into more or less obvious changes in phenotype. The mutagenic effect of any chemicals is most often attested by the ability to induce chromosomal restructuring in the mitotic anaphase and telophase of the root meristem and during meiotic division.

Table 1
Reaction mixture volume and final concentration of the reagents

| Reagent | Final volume (µL) | Final concentration |
|---|---|---|
| 5X Green GoTaq Flexi buffer | 5 | 1X |
| $MgCl_2$ 25 mM | 3 | 2 mM |
| dNTPs (nucleotid mixture) | 0.5 | 0.2 mM each dNTP |
| Primer 100 mM | 1.5 | 10 mM |
| GoTaq DNA polymerase (5 u/µL) | 0.25 | 1.25 u* |
| DNA | 2 | <10 ng/µL |
| Water up to 25 µL | 12.75 | - |

*1 unit (1u) is defined as the amount of enzyme required to catalyze the incorporation of 10 nmoles of nucleotids in 30 min at 74 °C

The analysis of a series of restructurings, including isochromatidic fragments, translocations, inversions, duplications, etc., requires thorough research on the metaphase.[29]

One of the most important elements for the estimation of cell survival and cytotoxicity exerted by some xenobiotic agents is the establishment of the mitotic index. When using SBPE and HSPE, the frequency of cells in mitosis is superior to that of the control, the maximum of this parameter being noted for the SB2 variant, which shows a mild mitogen effect (Table 3). In other works,[12,11] it was concluded that the SBPE in a concentration of 130 mg GAE/L determines the stimulation of growth in length and the increase of the amount of biomass accumulated in sunflower or maize plants. Therefore, the large number of cells in mitosis for SB2 and HS2 variants is correlated to the elongation of vegetative organs or increase in the amount of biomass. In another paper,[30] the action of aqueous extracts of *Trifolium repens* on root cells of *Vicia faba* was studied. The authors observed an increase in the mitotic index at low concentrations and a decrease of this parameter at high concentrations of the extract – data consistent with those obtained by us.

For the experimental variants, the percentage distribution of the four phases of mitotic division is similar to the control, meaning that they have the largest share in the prophase and metaphases. The anaphase and telophase achieve a similar pattern (Table 3). For the SB2 and HS2 variants, the mitotic index has the highest value. The growth rate of the cells in the prophase and the accumulation of cells in the anaphase and telophase can be attributed to delayed longitudinal cleavage of chromosomes and their migration to the two poles.

**Frequency of cells with aberrations**

Chromosomal aberrations were used as an indicator of plant sensitivity and were often correlated with morphological and taxonomic changes and the degree of sterility of plants can provide both quantitative data and qualitative information on exposure to different mutagens.[31] The importance of chromosome aberrations to the evolution, as well as their association with healthy vegetable and animal organisms, has been recognized for centuries, but their formation mechanisms are poorly known.[32,33]

Considering that any agent, physical or chemical, can exert clastogenic (producing chromosomal breaks) or turbagenic[34] effects (inducing mitotic or meiotic disturbances without directly affecting the DNA), we aimed to assess the involvement of the by-products used in this study in the division process. Therefore, determining the main types of aberrations and their frequency has been another objective of our study. The maximum of aberrations in ana-telophase for SB1 and HS1variants was recorded (Table 4). The presence of aberrations in division is correlated with the accumulation of a large number of cells in anaphase and telophase. An increase in the percentage of cells with aberrations is accompanied by their extended range. Thus, simple and multiple bridges, delayed or expelled chromosomes prevailed in the control sample. The treatment variants presented the ana-telophase (A-T) with bridges, delayed chromosomes and complex aberrations of type: A-T + bridges + delayed chromosomes and/or expelled, A-T multipolar + bridges (Table 4).

Since for SB2 and HS2 variants, the mitotic index achieved high values, the frequency of aberrations was close to that of the control sample. So, it can be concluded that low concentrations of SBPE/HSPE have positive effects on the division of cells. These effects can be attributed to the antioxidant properties of polyphenolic compounds, especially of catechine.[35] *In vitro* studies have demonstrated the antioxidant action of isoflavones present in extracts of red clover, namely of genistein, which inhibited the formation of hydrogen peroxide and superoxide anion.[36] In the case of daidzein, another isoflavone, contradictory results were reported: a number of studies indicated antioxidant properties,[37] however, in other experiments, daidzein caused oxidative stress at a cellular level.[38] Similar results were obtained[30] using red clover aqueous extracts. The authors found an intensification of the aberration rate in the root apex of *Vicia faba* as a function of the increase in extract concentration. The chromosomal aberrations observed included micronucleus, chromosomal fragments, bridges, and delayed chromosomes. Such observations were also recorded in the present study. At higher concentrations of the extracts, a higher number of cells with chromosomal aberrations were recorded, compared with those recorded at lower concentrations of the solutions tested.

Table 2
Characteristics of hemp shives and spruce bark polyphenolic extracts[17,14]

| Sample | Organic matter content (g/L extract) | pH (at 25 °C) | TPC (g /100g) | Catechins (g/100g) | Gallic acid (g/100g) | Vanillic acid (g/100g) | Caffeic acid (g/100g) | P-coumaric acid (g/100g) | Ferulic acid (g/100g) |
|---|---|---|---|---|---|---|---|---|---|
| HSPE | 0.42 ± 0.003 | 4.7 ± 0.2 | 16.1 ± 0.62 | 5.21 ± 0.23 | - | - | 9.68 ± 0.35 | 0.80632 ± 0.02 | 0.32598 ± 0.005 |
| SBPE | 0.42±0.04 | 4.7 ± 0.15 | 52±1.09 | 31 ± 1.9 | 3.19 ± 0.81 | 39.4 ± 0.2 | - | - | - |

Results reported represent the average values of triplicate determinations (n =3) ±S.E. TPC – Total polyphenolic contents

Table 3
Frequency of cells in interphase and mitotic division in the root apex of *Zea mays* L., SBPE and HSPE influence (%)

| Variant | Total cells in interphase | Total cells in division | Total cells in prophase | Total cells in metaphase | Total cells in anaphase | Total cells in telophase |
|---|---|---|---|---|---|---|
| Control | 89.06±0.07 [a] | 10.94±0.12 [b] | 5.21±0.02 [b] | 2.6±0.01 [a] | 1.25±0.00 [b] | 1.86±0.00 [b] |
| SB1 | 92.73±0.08 [a] | 7.27±0.06 [c] | 3.23±0.01 [c] | 1.12±0.01 [b] | 1.03±0.00 [b] | 1.82±0.00 [b] |
| SB2 | 81.75±0.03 [b] | 18.25±0.09 [a] | 9.79±0.02 [a] | 2.8±0.01 [a] | 2.36±0.01 [a] | 3.39±0.01 [a] |
| HS1 | 88.19±0.08 [a] | 11.81±0.14 [b] | 4.38±0.01 [c] | 2.5±0.01 [a] | 2.01±0.01 [a] | 2.87±0.01 [a] |
| HS2 | 82.18±0.15 [b] | 17.82±0.12 [a] | 10.13±0.02 [a] | 2.8±0.01 [a] | 2.11±0.01 [a] | 2.76±0.01 [a] |

Different letters within columns indicate significant differences ($p \leq 0.05$). Error bars indicate standard error of the mean ($n = 3$)

Table 4
Frequency of ana-telophase aberration types in the root apex of *Zea mays* L. under the influence of SBPE and HSPE (%)

| Variant | Total aberrations in ana-telophase | Bridges | | Lagging chromosomes | Expelled chromosomes | Anaphase with micronuclei | Telophase with micronuclei | Complex aberrations |
|---|---|---|---|---|---|---|---|---|
| | | 1 | n | | | | | |
| Control | 0.13±0.00 [d] | 0.01±0.00 [c] | 0.02±0.00 [c] | 0.05±0.00 [c] | 0.04±0.00 [c] | 0.00±0.00 [b] | 0.00±0.00 [c] | 0.01±0.00 [d] |
| SB1 | 1.28±0.01 [a] | 0.48±0.00 [a] | 0.25±0.00 [a] | 0.24±0.00 [a] | 0.14±0.00 [b] | 0.00±0.00 [b] | 0.00±0.00 [c] | 0.17±0.00 [a] |
| SB2 | 0.34±0.00 [c] | 0.11±0.00 [b] | 0.02±0.00 [c] | 0.08±0.00 [b] | 0.05±0.00 [b] | 0.01±0.00 [a] | 0.02±0.00 [b] | 0.05±0.00 [b] |
| HS1 | 0.72±0.00 [b] | 0.41±0.00 [a] | 0.00±0.00 [c] | 0.18±0.00 [b] | 0.00±0.00 [d] | 0.05±0.00 [a] | 0.05±0.00 [b] | 0.03±0.00 [c] |
| HS2 | 0.27±0.00 [c] | 0.05±0.00 [c] | 0.00±0.00 [c] | 0.05±0.00 [a] | 0.05±0.00 [a] | 0.00±0.00 [b] | 0.07±0.00 [a] | 0.05±0.00 [b] |

Different letters within columns indicate significant differences ($p \leq 0.05$). Error bars indicate standard error of the mean ($n = 3$)

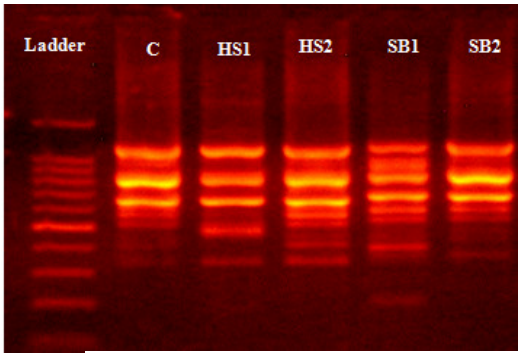


Figure 1: RAPD profile of maize DNA isolated from root apex influenced by SBPE and HSPE, using primer OPB 10 Marker - 100pb (C – control, SB1 – SBPE with 191 mg GAE/L, SB2 – SBPE with 130 mg GAE/L, HS1 – HSPE with 233 mg GAE/L, HS2 – HSPE with 164 mg GAE/L)

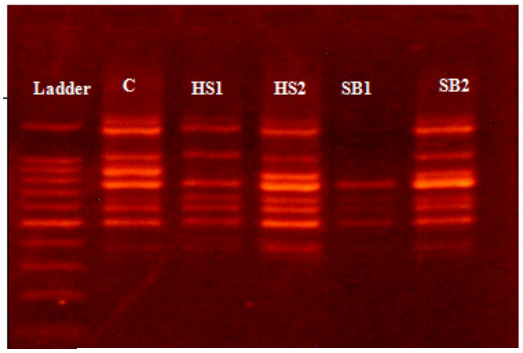


Figure 2: RAPD profile of maize DNA isolated from root apex influenced by SBPE and HSPE, using primer OPA 2 Marker - 100pb, (C – control, SB1 – SBPE with 191 mg GAE/L, SB2 – SBPE with 130 mg GAE/L, HS1 – HSPE with 233 mg GAE/L, HS2 – HSPE with 164 mg GAE/L

**Determination of DNA damage**

To identify changes at the DNA level, following the treatment with polyphenolic extracts, the RAPD method was used to obtain the characteristic profiles.

The amplification of DNA extracted from the root apex of the maize seedlings developed in growth media supplemented with different concentrations of polyphenolic aqueous extracts was carried out. Two primers were used as follows: primer OPB 10 Marker - 100bp and primer OPA 2 Marker - 100pb.

As may be seen in Fig. 1, the profiles are modified, which indicates a direct or indirect interaction of the polyphenols with DNA. A harmful effect on the cells from the root apex has been noted for the spruce bark polyphenolic extract with a concentration of 191 mg/L (SB1). The profiles show a few bands of low intensity and a maximum length of 600 base pairs. It can be seen for SBPE that the intensity of the bands decreases at a higher concentration of the extract (SB1). This observation can be attributed to the appearance or disappearance of merged sites of the primers. While the profile of SB2, when the concentration of the polyphenolic compounds was 130 mg/L, does not seem to have drastically changed, the growth profile of SB1 (191 mg/L) appears much weaker, because of the large number of changes to the nucleotide sequence (Fig. 2).

Regarding the influence of HSPE, analyzing Figures 1 and 2, it may be noted that the bands of HS1 have lower intensity, and are fewer, compared to the control. Instead, the bands for HS2 (where the concentration of the extract applied was lower – 164 mg GAE/L) are more numerous and have higher intensity, compared to the bands of C (control). In addition, Fig. 1 (amplified with the primer OPB 10 Marker - 100pb) shows that the bands of HS1 and HS2 are more intense and well defined, compared to the control. The same observation was made in the case of the amplification with the primer OPA 2 Marker - 100bp (Fig. 2).

The RAPD method allowed observing that the effect generated by the SBPE and HSPE in high concentrations was the inactivation of DNA. In contrast, at lower concentrations the amount of DNA was higher, as evidenced by more intense and more numerous bands compared to the control.

**CONCLUSION**

When using SBPE or HSPE, the frequency of cells in mitosis is superior to that of the control. The maximum of this parameter was found in the variant with the lowest concentration of polyphenolic compounds (SB2-130 mg GAE/L), highlighting slight mitosis.

At the higher concentrations of the extracts used, there were a higher number of cells with chromosomal aberrations, compared with that the number of cells recorded at lower concentrations of the solutions tested. A significant proportion of the treatment variants presented the ana-telophase (A-T) with bridged, delayed chromosomes and complex aberrations of type A-T + bridge + delayed and/or expelled chromosomes, A-T multipolar + bridges.

Thus, it can be concluded that low concentrations of the aqueous extracts obtained from spruce bark or hemp shives have positive effects, which can be attributed to their antioxidant properties: the amount of DNA is high, evidenced by more intense and more numerous bands compared to the control. However, high concentrations of the spruce bark and hemp shives extracts can lead to the inactivation of DNA.

## ABBREVIATIONS

SBPE – spruce bark polyphenolic extract, HSPE – hemp shives polyphenolic extract, TPC – total polyphenolic content, RAPD – Random Amplified Polymorphic DNA, GAE – equivalents of gallic acid, ANOVA – analysis of variance.

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