Canadian Journal of Pure and Applied Sciences
Vol. 10, No. 1, pp. 3721-3726, February 2016
Online ISSN: 1920-3853; Print ISSN: 1715-9997
Available online at www.cjpas.net

DESMUTAGENIC AND ANTIMUTAGENIC POTENTIAL OF *EHRETIA WALLICHIANA* HOOK.F. AND THOMSON EX GAMBLE

*Fatma Hashem[1], Hemaia Motawea[1], Manal Shabana[2], Maysa Elsayed Moharam[3], Mai Khalil[1] and Taha Al-Alfy[4]
[1]Department of Pharmacognosy, National Research Centre
[2]Department of Phytochemistry and Plant Systematic, National Research Centre
[3]Department of Microbial Chemistry, National Research Centre
[4]Department of Pharmacognosy, Faculty of Pharmacy, Cairo University, Egypt

ABSTRACT

The ethyl acetate, ethanolic extract and total ethanol extract of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble were tested for desmutagenic and antimutagenic activity. All of them have antimutagenic activity but lack desmutagenic activity. Ethyl acetate has highest activity as free radical scavenger (97.6%) in comparison with other extracts followed by total ethanol extract (88.7%), which may be attributed to flavonoids and other phenolics in the extracts. Betulinic acid, 9-hydroxy-10, 12, 15 octadecatrienoic acid methyl ester, Rosmarinic acid and ethyl rosmarinate were isolated from ethanolic extract. This is the first report of rosmarinic acid and ethyl rosmarinate isolated from *Ehretia wallichiana*. This is the first report of ethyl rosmarinate in genus *Ehretia*.

Keywords: *Ehretia wallichiana*, desmutagenic activity, antimutagenic activity, rosmarinic acid and ethyl rosmarinate.

INTRODUCTION

Ehretia was named after the 18th century botanical artist, Ehret (www.plantzafrica.com). The genus *Ehretia* is comprised of about 50 species of evergreen or deciduous shrubs and trees of tropical and subtropical regions of both the New and Old World (Bailey and Bailey, 1976). They exist mainly in Africa and South Asia, three in North America and the Caribbean and 14 species in China mainly south of the Chang Jiang. Some species are known as *Cordia.* (www.efloras.org). The wood of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble is used for building, charcoal and for tea-boxes (www.powerhousemuseum.com).

Preparation of successive extracts

For preparation of experiment, 1 kg of the air-dried powdered leaves of the plant was extracted with solvents of increasing polarities: petroleum ether then chloroform then ethyl acetate and 95% ethanol in a Soxhlet apparatus. After complete extraction, these extracts were evaporated to dryness under vacuum at 40°C.

Preparation of total ethanol fraction

The powdered air-dried leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble (500g) was extracted with 80% ethanol in a Soxhlet apparatus.

*Corresponding author e-mail: maiikhalil@hotmail.com

Investigation of successive extracts

The chloroform extract contains terpenes, the ethyl acetate and ethanol extracts gave positive tests for flavonoids, tannins and glycosides. These extracts were examined by paper chromatography using paper Whatmann No. 1 sheets with the two developing systems (a) *n*-butanol - acetic acid - water (4:1:5 v/v organic upper layer) and (b) acetic acid - water (15:85 v/v). The chromatograms were examined under UV light.

Isolation of successive extracts

Isolation of ethyl acetate and ethanol extracts was carried out by PPC using Whatmann No. 3MM sheets and the two solvent systems: a and b. After development, marker bands were visualized under UV light. Four compounds were isolated from ethanol. The compounds were further purified on Sephadex LH-20 column using methanol as eluent.

Compound 1:
R_f 0.4 in (a) and 0.72 in (b).
UV: λ_{max} (MeOH): 280 nm.
IR: 3758.5, 2925.4, 2852.2, 1625.7, 1384.6, 1122.3 and 1072.2cm^{-1}.
MS: m/z (relative abundance) 273 (2%), 229 (3%), 169 (3%), 127 (6%), 99 (10%), 85 (27%), 71 (55%), 57 (100%), corresponding to molecular formula $C_{30}H_{48}O_3$.
^{1}H-NMR (500 MHz;CD_3OD): δ in ppm:9.6 (s, OH carboxylic), 4.6 and 4.8 (s, terminal CH_2), 3.2 (dd,

J=11.2 and 5.2 Hz, H-3), 2.04 (s, OH hydroxyl),from 1.26 to 3 ppm characteristic hump of sterol-terpene CH_2 and 0.87, 0.87, 0.91, 0.92, 1.26 and 1.29 (s, six methyl).

Compound **2**:
R_f 0.5 in (a) and 0.3 in (b).
UV: λ_{max} (MeOH): 275 nm.
IR: 3434.6, 2920.6, 1632.4 and 1427cm^{-1}.
MS: m/z (relative abundance) 308 (M^+, 1%), 114 (100%), 91 (49%), 85 (26%), 69 (80%), 57 (84%), corresponding to molecular formula $C_{19}H_{32}O_3$.
^{1}H-NMR (500 MHz ; $(CD_3)_2CO$): δ in ppm: 5.5 and 5.2 (s, H-10 and H-11), 3.98 (s,OCH3) hidden by water peak,2.0 (s,OH) and hump from 1.5 to 2.02 for CH_2 group.

Compound **3**:
Colorless amorphous powder.
R_f values (PC): 0.65 in (a), 0.3 in (b).
It gave blue spot under UV which was intensified upon NH_3 vapours exposure.
UV (MeOH) λ_{max}: 285, 335 nm.
IR: 3429.7, 2926.4, 1731.7, 1631.4, 1460.8, 1280.5, 1128.1, 1048.1 cm^{-1}.
^{1}H-NMR (400 MHz; DMSO,d_6) δ: 7.40 (1H, d, *J*= 16 Hz, H-7'), 7.00 (1H, d, *J*= 2 Hz, H-2'), 6.90 (1H, dd, *J*= 8&2 Hz, H-6'), 6.75 (1H, d, *J*= 8 Hz, H-5'), 6.62 (1H, d, *J*= 2 Hz, H-2) 6.55 (1H, d, *J*= 8.0 Hz, H-5), 6.47 (1H, dd, *J*= 8&2 Hz, H-6), 6.15 (1H, d, *J*= 16 Hz, H-8'), 4.80 (1H, dd, *J*= 7.5&3.3 Hz, H-8), 2.99 (1H, dd, *J*= 14&3.3 Hz, H-7a), 2.70 (1H, dd, *J*= 14&7.5 Hz, H-7b).

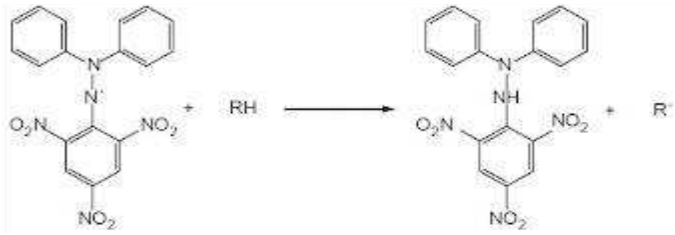
Compound 4:
Colorless amorphous powder.
R_f values (PC): 0.6 in (a), 0.32 in (b).
It gave fluorescent blue spot under UV which was intensified upon NH_3 exposure.
UV (MeOH) λ_{max}: 286.6, 327.8 nm.
IR: 3420.1, 2925.4, 1728.8, 1655.5, 1457.9, 1278.5 ,1128.1, 1073.1 cm^{-1}.
^{1}H-NMR (400 MHz; DMSO,d_6) δ: 7.40 (1H, d, *J*= 16 Hz, H-7'), 7.00 (1H, d, *J*= 2 Hz, H-2'), 6.90 (1H, dd, *J*= 8&2 Hz, H-6'), 6.75 (1H, d, *J*= 8.0 Hz, H-5'), 6.70 (1H, d, *J*= 8 Hz, H-2), 6.60 (1H, d, *J*= 8 Hz, H-5), 6.50 (1H, dd, *J*= 8&2 Hz, H-6), 6.20 (1H, d, *J*= 16 Hz, H-8'), 4.90 (1H, dd, *J*= 8&3.3 Hz, H-8), 4.20 (2H, m, $COOCH_2CH_3$), 3.05 (1H, dd, *J*= 14&3.3 Hz, H-7a), 2.75 (1H, dd, *J*= 14&8 Hz, H-7b), 0.85 (3H, m, $COOCH_2CH_3$).

MATERIALS AND METHODS

SCREENING *IN-VITRO* ANTIOXIDANT ACTIVITY

PROCEDURE

The free radical scavenging activity of successive extracts: petroleum ether, chloroform, ethyl acetate, 95% ethanol and total 80% ethanol was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of (Shimada *et al*., 1992). All plant extracts were screened at 100 µg/ mL while the most potent active extracts (gave more than 90%) were assayed at 25-75µg/ mL. In a flask, 1 mL of 0.1mM solution of DPPH in methanol was added to 3 mL of extract solution at different conc. (25-75µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in Asysmicroplate reader. The reaction mixture with lower absorbance indicate higher free radical scavenging activity. DPPH scavenging effect (%) = 100 − [((A0-A1)/A0) × 100]. Where A0 was the absorbance of the control reaction and A1 was the absorbance in the presence of the sample (Oktay *et al*., 2003).
Chemical reaction:



1,1-diphenyl-2-picrylhydrazyl ⟶ 1,1-diphenyl-2-picrylhydrazine

$$(DPPH^{\bullet}) + (H—A) \rightarrow DPPH—H + (A^{\bullet})$$
Purple ⟶ Yellow

Inhibition of mutagen-induced revertants in *Salmonella typhimurium*

Desmutagenic and antimutagenic activities of leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble extracts were detected by using the bacterial strain *Salmonella typhimurium* TA100 (His^-). The concentration of each mutagen used was: (25µL in 100µL DMSO). EMS (Ethylmethyl sulphonate), RL (Ribose lysine): (0.5 M in water) as chemical mutagen (McCann *et al*., 1975a, b). Ascorbic acid was used as reference desmutagenic and antimutagenic compound (Bijur *et al*., 1997).

Desmutagenic activity

Various amounts of the ethyl acetate, ethanol extract and total ethanol extract, Ascorbic acid as reference drug and the mutagen were added to sterile distilled water (1mL final volume) containing 100 µmol. phosphate buffer (pH 7.4). The mixture was incubated at 37°C for 30 min. 100 mL of a 24 hours bacterial culture of TA100 His^- strain (10^8 cells) and 2mL of molten top agar (45°C) were added and the mixture was poured onto minimal glucose-agar plates: After addition of the cells the mixture was incubated once more for 1/2 hour at 37°C. The number of

His^+ induced revertants were scored after incubation for 48 hours at 37°C.

Antimutagenic activity

Various concentrations of ethyl acetate, ethanol extract and total ethanol extract were added to sterile distilled water (1mL final volume) containing 100µL of 24 hours culture of TA100 His^- test strain and 100g mol. phosphate buffer pH 7.4. After incubation at 37°C for 1/2 hour, cells were collected by centrifugation, washed twice with phosphate buffer to remove antimutagen (tested compounds) and finally suspended in 1 mL of the buffer. After addition of the mutagen and 2mL of soft agar, the mixture was poured onto minimal glucose agar plates. After incubation for 48 hours at 37°C revertant colonies (His^+) were counted. Desmutagenic and antimutagenic activities were calculated as the percentage of decrease of induced revertants according to (Hayatsu *et al.*, 1988) and (Mortelmans and Zeiger, 2000) after subtraction of corresponding spontaneous reversion according to the equation:

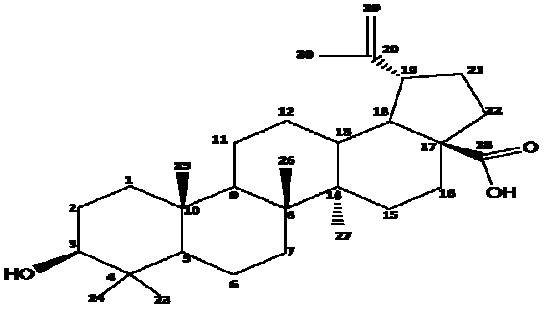
% inhibition = 100 – (N / N_0 X 100), N is the revertant / plate induced by the mutagen in the presence of increasing amounts of the tested material. N_0 is the reversion induced in the control. The results are illustrated in Table 3.

RESULTS AND DISCUSSION

Compound **1:**

The structure of compound **1** was identified on the basis of its spectral data (UV, ^{1}H-NMR and EI-MS) which were identical with betulinic acid.

The positive EI-MS showed the characteristic fragments of sterols and terpenes such as 273, 229, 169, 127, 99, 85 and 71. This confirmed the structure corresponding to molecular weight (M^+) 456 and molecular formula ($C_{30}H_{48}O_3$) of betulinic acid. ^{1}H-NMR shows six methyl at 0.87, 0.87, 0.91, 0.92, 1.26 and 1.29 while OH carboxylic at 9.6, OH hydroxyl at 2.04, H-3 at 3.2, terminal CH_2 at 4.6 and 4.8, characteristic hump of sterol-terpene CH_2 from 1.26 to 3 ppm. Compound **1** was found to be betulinic acid (Chowdhury *et al*., 2013). It was isolated before from *Ehretia laevis* (Dan and Dan, 1982).

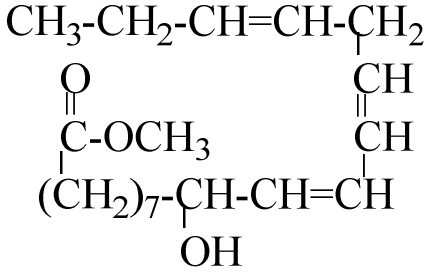


betulinic acid

Compound **2:**

The structure of compound **2** was identified on the basis of its spectral data (UV, ^{1}H-NMR and EI-MS) which were identical with9-hydroxy-10,12,15-octadecatrienoic acid methyl ester.

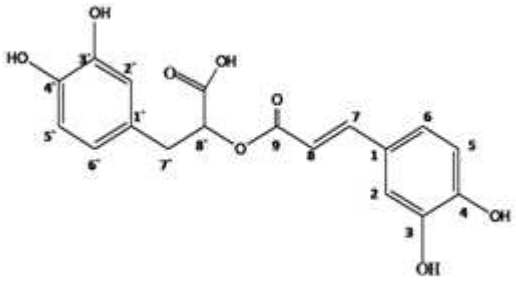
The positive EI-MS confirmed the structure and gave signal at m/z 308(M^+) corresponding to molecular formula ($C_{19}H_{32}O_3$) of 9-hydroxy-10,12,15-octadecatrienoic acid methyl ester. Compound **2** was found to be 9-hydroxy-10,12,15-octadecatrienoic acid methyl ester isolated before from *Ehretia dicksonii* (Dong *et al*., 2000).



9-hydroxy-10,12,15-octadecatrienoic acid methyl ester.

Compound **3** data confirmed that it was rosmarinic acid. Absorption maxima of UV spectra were 285 and 335 nm as reported for this compound in the literature (Mehrabani *et al.*, 2005). The IR spectrum showed OH groups at 3429.7 cm^{-1} and carbonyl at 1731.7 $cm^{-1}$and aromatic rings at 1460.8 cm^{-1}. ^{1}H-NMR data of the compound confirmed the structure of rosmarinic acid as reported in the literature (Mehrabani *et al.*, 2005).

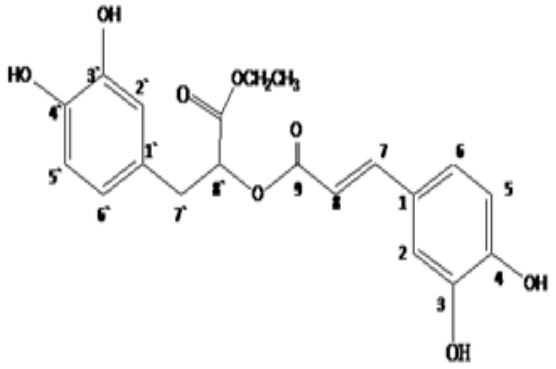
Rosmarinic acid is found in plants *Lithospermum erythrorhizon*, *Anchusa officinalis* and *Ehretia thyrsiflora* from the Boraginaceae family (Misawa, 1997; Yamamoto *et al.*, 2000; Li *et al.*, 2009). Rosmarinic acid was first isolated from *Rosmarinus officinalis* (Scarpati and Oriente, 1958).



Rosmarinic acid.

Compound **4** showed absorption maxima of UV spectra at 286.6 and 327.8 nm. The IR spectrum showed OH groups at 3420.1 cm^{-1}, carbonyl at 1728.8 cm^{-1} and aromatic rings at 1457.9 cm^{-1}. The

^{1}H-NMR data of compound **4** was similar to compound **3** in addition to signals at δ 4.20 (m, 2H) and 0.85 ppm (m, 3H). These two signals were attributed to an ethyl group esterifying the rosmarinic acid confirming that compound **4** was ethyl rosmarinate (Hou *et al.,* 2002) which is isolated for the first time from this genus.



Ethyl rosmarinate

The results obtained from absorbance of DPPH by UV are illustrated in Table 1 and 2 and it could be concluded that ethyl acetate has highest activity as free radical scavenger (97.6%) in comparison with other extracts followed by total ethanol extract (88.7%), which may be attributed to flavonoids and other phenolics in the extracts. This agrees with the results obtained by (Torane *et al.,* 2011) who studied the antioxidant activity of leaves and stem of *Ehretia laevis* reporting that the leaves have higher antioxidant activity than the stem. The high scavenging activity may be due to hydroxyl groups existing in the phenolic compounds.

Table 1. DPPH radical scavenging activity of successive extracts and total ethanol extract of leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble.

Extract	% of scavenging effect	IC_{50}(µg/ mL)	IC_{90}(µg/ mL)
Petroleum ether	15	----	----
Chloroform	28.3	----	----
Ethyl acetate	97.6	35	64.2
95% Ethanol	8.5	----	----
Total 80% ethanol	88.7	60.3	95.9

IC50 of ascorbic acid was 4.8 µg/ mL

Table 2. DPPH radical scavenging activity of ethyl acetate extract and total ethanol extract of leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble.

Concentration (µg/ mL)	% of scavenging effect of ethyl acetate extract	% of scavenging effect of total ethanol
100	97.6	88.7
50	76.9	42.3
25	45.3	16.1
12.5	19.4	2.3
6.25	6.7	0

Desmutagenic and antimutagenic activities of leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble extracts were ascertained by measuring the inhibition of TA 100 His^+ revertants induced by ethyl methane sulphonate (EMS) and ribose lysine (RL). The results, represented in Table 3 showed that %inhibition of total ethanol extract is higher antimutagenic, especially in case of RL. Tests on RL induced reversion adverted on good activity of all the extract specimens. Different concentrations of the three extracts showed negative desmutagenic activity induced by both mutagens.

The antimutagenic factors are divided into two main classes: desmutagen and antimutagen according to differences in their modes of action. Desmutagens in activates or destroys mutagens directly or indirectly out of the cell and pre-incubation treatment is designed to evaluate the desmutagenic effect. The other type is called a bio- antimutagen, which suppresses the process of mutagenesis itself in the cell, for example, it eliminates radicals or increases DNA repair systems, other antimutagens that exert its effect by acting as blocking agents (Hung *et al*., 2009). Recent research has confirmed that plant flavonoids inhibit the mutagencity induced by chemical mutagens. (Miyazawa and Hisama, 2003).

The antimutagenic or protective effect has been attributed to many classes of phytocompounds mainly flavonoids and phenolic compounds present in foods. Phenolic compounds have also been reported to exhibit a wide range of other biological activities such as antimicrobial, anti-inflammatory, antioxidant and free radical scavenging (Negi *et al*., 2003; Shon *et al*., 2004).

Table 3. Desmutagenic and antimutagenic potential of leaves of *Ehretia wallichiana* Hook.f. & Thomson ex Gamble extracts using EMS and RL induced revertants.

Sample	Conc. µg/plate	Desmutagenic % inhibition		Antimutagenic % inhibition	
		EMS	RL	EMS	RL
Ethyl acetate	40	0	0	0	40.00
	80	0	0	46.15	63.33
	100	0	0	46.15	76.67
Ethanol	40	0	0	57.69	0
	80	0	0	76.92	70.00
	100	0	0	0	58.33
Total ethanol	40	0	0	61.54	0
	80	0	0	65.38	86.67
	100	0	0	26.92	10.00
Ascorbic acid	40	96	57	67	0
	80	97	86	99	0
	100	100	87	100	0

EMS: Ethyl methylsulphonate RL: Ribose lysine

REFERENCES

Bailey, LH. and Bailey. 1976. EZA Concise Dictionary of Plants Cultivated in the United States and Canada. (1st edi.). 418. Macmillan Publishing Company Inc., New York and Collier Macmillan Publishers, London.

Bijur, GN., Ariza, ME., Hitchcock, CL. and Williams, MV. 1997. Antimutagenic and promutagenic activity of ascorbic acid during oxidative stress. Environ. Mol. Mutagen. 30(3):339-45.

Chowdhury, A., Alam, Md. A., Rashid, RB., Al-Mansur, MA., Rahman, MS. and Rashid, MA. 2013. Steroids and triterpenoids from *Corypha taliera* Roxb: a critically endangered palm species of Bangladesh. Research Journal of Medicinal Plant. 7:125-129.

Dan, S. and Dan, SS. 1982. Triterpenoids of the bark of *Ehretia laevis*. Fitoterapia. 53:51-52.

Dong, M., Oda, Y. and Hirota, M. 2000. (10*E*,12Z,15Z)-9-hydroxy-10,12,15-octadecatrienoic acid methyl ester as anti-inflammatory compound from *Ehretia dicksonii*. Biosci. Biotech. Bioch. 64:882-886.

Hayatsu, H., Arimoto, S. and Negishi, T. 1988. Dietary inhibitors of mutagenesis and carcinogenesis. Mut. Res. 202: 429-446.

Hou, ZF., Tu, YQ. and Li, Y. 2002. Three new phenolic compounds from *Nepeta prattii*. Journal of the Chinese Chemical Society. 49:255-258.

Hung, YH., Wang, YJ. and Chou, CC. 2009. Antimutagenic activity of *Aspergillus awamori*-fermented black soyabean response to stimulated digestive juice treatments and its antimutagenic mechanisms. LWT-Food Sci. Technol. 42:56-62.

Li, L., Peng, Y., Xu, LJ., Li, MH. and Xiao, PG. 2009. Flavonoid glycosides and phenolic acids from *Ehretia thyrsiflora*. Biochem. Syst. Ecol. 36:915-918.

McCann, J., Spingarn, N.,Kobori, J. and Ames, B. 1975[a]. Detection of carcinogens as mutagens: Bacterial tester strains with R factor plasmids. Proc. Natl. Acad. Sci. USA.72: 979-983.

McCann, J., Choi, E., Yamasaki, E. and Ames, B. 1975[b]. Detection of carcinogens as mutagens in the *Salmonella* micro some test: Assay of 300 chemicals. Proc. Nati. Acad. Sci. USA.72:5135-5139.

Mehrabani, M., Ardakani, MS., Alireza Ghannadi, A., Dehkordi, NG. and Seyyed Ebrahim Sajjadi Jazi, SES. 2005. Production of Rosmarinic Acid in *Echium*

amoenum Fisch. and C.A. Mey. Cell Cultures. Iranian Journal of Pharmaceutical Research. 2:111-115.

Misawa, M. 1997. Plant tissue culture: an alternative for production of useful metabolites. 60-62. Daya Publishing House, Dehli, India.

Miyazawa, M. and Hisama, M. 2003. Antimutagenic activity of flavonoids from *Chrysanthemum morifolium*. Biosci Biotechnol Biochem. 67:2091-2099.

Mortelmans K. and Zeiger, E. 2000. The Ames *Salmonella* microsome mutagenicity assay. Mut. Res. 455:29-60.

Negi, PS., Jayaprakash, GK. and Jena, BS. 2003. Antimutagenic and antioxidant activities of pomegranate peel extract. Food Chem. 80:393.

Oktay, M., Gülçin, I. and Küfrevio, I. 2003. Determination of in vitro antioxidant activity of fenne (*Foeniculum vulgare*) seed extracts. Lebensmittel-Wissenchaft und Technologie. 36-263.

Scarpati, ML. and Oriente, G. 1958. Isolamento e costituzione dell'acido rosmarinico (dal *Rosmarinus off.*) (Italian). Ric Sci. 28:2329-2333.

Shimada, K., Fujikawa, K., Yahara, K. and Nakamura, T. 1992. Antioxidative properties of xanthine and autoxidation of soybean oil in cyclodextrin emulsion. Journal of Agriculture and Food Chemistry. 40: 945-948.

Shon, MY. Choi, SD., Kahng, GG., Nam, SH. and Sung, NJ. 2004. Antimutagenic, antioxidant and free radical scavenging activity of ethyl acetate extracts from white, yellow and red onions. Food Chem. Toxicol. 42(4):659.

Torane, RC., Kamble, GS., Khatiwora, E., Ghayal, NA. and Deshpande NR. 2011. Antioxidant Capacity of Leaves and Stem of *Ehretia laevis*. International Journal of Pharmacy and Pharmaceutical Sciences. 3(2): 149-151.

Yamamoto, H., Inoue, K. and Yazaki, K. 2000. Caffeic acidoligomers in *Lithospermum erythrorhizon* cell suspension cultures. Phytochemistry. 53:651-657.

Received: Dec 4, 2015; Revised: Dec 16, 2015;
Accepted: Dec 19, 2015