

Essential Oil Composition and *in vitro* Antioxidant Activity of Ethanolic Extract of *Thymus daenensis* Celak from Iran

^{1,2}Zahra Sabahi, ³Mohammad M. Zarshenas, ⁴Fatemeh Farmani, ³Pouya Faridi, ^{5,6}Soheila Moein and ^{4,7}Mahmoodreza Moein,

¹Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz, University of Medical Sciences, Shiraz, Iran

²Pharmaceutical Sciences Research Center, School of Pharmacy, Shiraz, University of Medical Sciences, Shiraz, Iran

³Department of Traditional Pharmacy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

⁴Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran

⁵Department of Biochemistry, School of Medicine, Hormozgan University of Medical Sciences, BandarAbbas, Iran

⁶Research Center for Molecular Medicine, School of Medicine, Hormozgan University of Medical Sciences, BandarAbbas, Iran

⁷Medicinal Plants Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract: The genus *Thymus* (Lamiaceae) family possesses different species in Iran. In this study, the antioxidant potential of essential oil and crude extract of one of the endemic species of *Thymus*, *Thymus daenensis* were evaluated. For analysis antioxidant potency, nitric oxide, DPPH free radical, hydroxyl radical scavenging, ferric reducing antioxidant power assay and inhibition of lipid peroxidation were used. The antioxidant potential of crude extract by DPPH free radical scavenging ($IC_{50} = 194 \pm 0.02 \mu\text{g/mL}$), nitric oxide scavenging ($74 \pm 0.0\%$) and hydroxyl radical scavenging ($60.2 \pm 0.27\%$) was more active than essential oil. In nitric oxide scavenging crude extract was more active than the antioxidant standard BHT, 42 ± 0.79 . Inhibition of lipid peroxidation of essential oil and crude extract of *Thymus daenensis* were lower than BHT and Vit C. The essential oil of *Thymus daenensis* showed no antioxidant potency by ferric-reducing antioxidant power (Frap) assay but the extract possesses antioxidant activity ($10.16 \pm 1.6 \mu\text{mol/g}$) by this method. The profile of essential oil was detected by GC-Mass. The main component of essential oil was Geraniol (66.8%), Geranyl acetate (13.9%) and Beta caryophyllene (9.6%).

Key words: Antioxidant potential • Crude extract • Essential oil • GC-mass • *Thymus daenensis*

INTRODUCTION

The genus *Thymus* (Lamiaceae) is represented by different species in Iran and 6 of them are endemic in Iran [1]. Thyme is used as an analgesic and anti-inflammatory in traditional Persian medicine and also is used as antitussive, antifungal, antiviral, antibacterial, anthelmintic, antispasmodic, carminative, sedative, radical

scavenging as well as antioxidant agent [2]. Phytochemical analysis of *Thymus* spp. components showed that this plant contains terpenoids, flavonoid aglycones, flavonoid glycosides and phenolic acids. *Thymus daenensis* (Td) is one of those endemic species in Iran and is widely used as medicine and food additive. Few studies showed its antioxidant and its essential oil profile [3]. Usage of natural antioxidant prevents different

Corresponding Author: Mahmoodreza Moein, Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.
Fax: +98 711 2424126.

diseases such as diabetes, cancer and atherosclerosis. But usage of synthetic antioxidants such as Butylated Hydroxy Toluene (BHT) and Butylated Hydroxy Anisole (BHA), have side effects.

Thus today's attentions attributed to natural antioxidants which had fewer side effects.

Therefore this investigation deals with the detailed analysis of the essential oil of Td and also undertaken to determine the antioxidant and radical scavenging properties of *Thymus daenensis*.

MATERIALS AND METHODS

Chemicals: Butylated hydroxy toluene (BHT), gallic acid, quercetin, DPPH (2, 2-diphenyl-1-picrylhydrazyl), TPTZ (2, 4, 6-tripyridyl-S-triazine) were purchased from Sigma (ST. Louis, MO, USA). All other reagents were purchased from Merck Chem. Co.

Plant Material: Aerial parts of Td were collected from Sepidan (north part of Fars province of Iran) in June 2010. Plant material was identified by Miss. S. Khademian and a voucher specimen number was deposited at Herbarium of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Essential Oil Preparation: Aerial parts of Td (39g) were subjected to hydrodistillation in order to yield (0.9 ml) essential oils. Total essential oil was subsequently analyzed by GC/MS. GC/MS analysis was carried out using a Hewlett-Packard 6890/5973 operating at 70.1 eV ionization energy, equipped with a HP-5 capillary column (phenyl methyl siloxane, 25 m × 0.25 mm i.d) with He as the carrier gas and split ratio, 1:20. Oven temperature was performed as follows: 60°C (3 min) to 260°C at 3°C/min; detector temperature, 260°C; carrier gas, He (0.9 mL/min). Retention indices were determined by using retention times of n-alkanes that were injected following essential oil under the same chromatographic conditions. The components of the oils were identified by comparison of their mass spectra and retention indices (RI) with those given in literature as well as comparison of their mass spectra with the Wiley library or with the published mass spectra [4,5].

The radical scavenging effects of dried extract were evaluated using DPPH and NO radical scavenging procedure. Also, ferric-reducing antioxidant power assay (FRAP) was carried out by TPTZ.

DPPH Free Radical Scavenging Assay: The power of scavenging the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was measured in a modified assay [6].

DPPH radical (100mM) in methanol was mixed with various concentrations (1.6- 3200 µg/mL) of methanol extract (100 µL). The reaction mixture was incubated at room temperature for 30 min in the dark. The DPPH radical inhibition was measured at 490 nm by using a microplate reader. The IC₅₀ of each sample, concentration of extract scavenge 50% DPPH radicals, was calculated. All measurements were performed in triplicates. Averages and standard deviations are calculated.

The antioxidant activity (AOA) was given by:

$$100 - [(A) \text{ sample} - (A) \text{ blank}] \times 100 / (A) \text{ control}].$$

“A”: the absorbance of the color formed in microplates wells.

Control: Contains DPPH without plant extract, blank: contains only methanol.

Nitric Oxide Radical Scavenging Assay: Nitric oxide (NO) radical scavenging method was determined according to previous study with some modifications [7,8]. Fifty µL of 10 mM sodium nitroprusside in phosphate buffer (0.2 M, pH 7.4) was mixed with 50 µL of each sample and incubated at 27°C for 150 min. After incubation, 100 µL of Griess reagent was added to each sample and then incubated at a room temperature for 5 min. Finally, absorbance was measured at 542 nm [7].

Nitric oxide radical scavenging was determined as follow:

$$[(A)_{\text{control}} - A_{\text{sample}}] / A_{\text{control}} \times 100$$

Blank: Absorbance of extract without any reagent

Control: Absorbance of control without sample

FRAP (Ferric-Reducing Antioxidant Power) Assay: TPTZ (2, 4, 6-tripyridyl-S-triazine) solution (10 mmol L⁻¹) in HCl (40 mmol/ L), FeCl₃ (20 mmol/ L), acetate buffer (0.3 mol/ L, pH 3.6) were the solutions needed for this method. Acetate buffer, FeCl₃ and TPTZ were mixed before usage. This mixture heated to 37°C, 20 µL of each sample and 180 µL of FRAP reagent were mixed in a 96-well microplate reader then incubated at 37°C for 10 minutes. The absorbance of complex measured at 593nm. This protocol relieve to previous method with slight modifications [9].

Antioxidant Activity Assay (Ferric Thiocyanate Method):

This assay was carried out as explained in the method of Kikuzaki and Nakatani with slight modifications [10] At first a mixture should be ready in a screw-cap vial which contains: 4.0 mg of a sample in 4 mL of 99.5% ethanol, 4.1 mL of 2.5% linoleic acid in 99.5% ethanol, 8.0 mL of 0.02 M phosphate buffer (pH 7.0) and 3.9 mL of water then this mixture was placed at 40°C in the dark.

To 0.1 mL of this mixture, 9.7 mL of 75% (v/v) ethanol and 0.1 mL of 30% ammonium thiocyanate was added. After that 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid was added to the reaction mixture, the absorbance was measured at 500 nm. The absorbance was measured at this wavelength every 24 hours until the day after that the absorbance of the control reached its maximum value.

Determination of Hydroxyl (OH) Radical Scavenging Activity:

The hydroxyl radical scavenging activity of Td extract and essential oil were measured by the deoxyribose method [11] and compared with gallic acid and BHT. The reaction mixture contain (final concentration 15.62–1000 µg/mL of Td extract or essential oil), 200 µl deoxyribose (15 mM), 100 µl H₂O₂ (10 mM), 200 µl phosphate buffer (0.1M, pH 7.4), 200 µl FeCl₃ (5 mM), 100 µl EDTA (10 mM) and 100 µl ascorbic acid (10 mM) which were incubated in a water bath at 37 ± 0.5°C for 1 h. Then the TBA (1 mL, 1% w/v) and TCA (1 mL, 2.8%) were added to the mixture and heated in a water bath at 100 °C for 20 min, the absorbance of samples were measured in 532 nm [11]. BHT and gallic acid were used as standards.

Control contains all of the solutions, except plant extract, distilled water used as blank.

Inhibition of deoxyribose degradation in percent was calculated according to the equation:

$$(A_1 - A_2) / A_1 \times 100$$

A₁: the absorbance of control

A₂: the absorbance of test

RESULTS

The main constituents of the essential oil were identified as Geraniol (66.8%), Geranyl acetate (13.9%) and Beta-Caryophyllene (9.6%). No trace of thymol was found in essential oil analysis. In DPPH radical scavenging, the IC₅₀ of Td essential oil and extract was higher than antioxidant standards (Table.1). Also the IC₅₀ of Td extract (194.24±0.021 µg/ mL) was lower than the IC₅₀ of Td essential oil (>3200 µg/ mL). Nitric radical scavenging of Td extract (74%) was higher than essential oil (35%) and BHT (42%), Table 1. In FRAP method the Td extract was determined as 10.16 ± 1.6 µmol/g (Table 1). In FTC method, the Td extract and Td essential oil were (46.04 ± 0.24) and (48.33 ± 0.12) respectively, which lower than BHT (66.86±0.15) and quercetin (64.06±0.02), Table 1. In hydroxyl scavenging the Td extract (60.21±0.27) was higher than Td essential oil (54.71 ± 0.17), Table 1. Antioxidant standards such as ascorbic acid (64.06± 0.2) and BHT (66.86± 0.15) inhibited lipid peroxidation higher than essential oil (54.7±0.17) as well as extract of Td (60.2 ± 0.27), Figure 1.

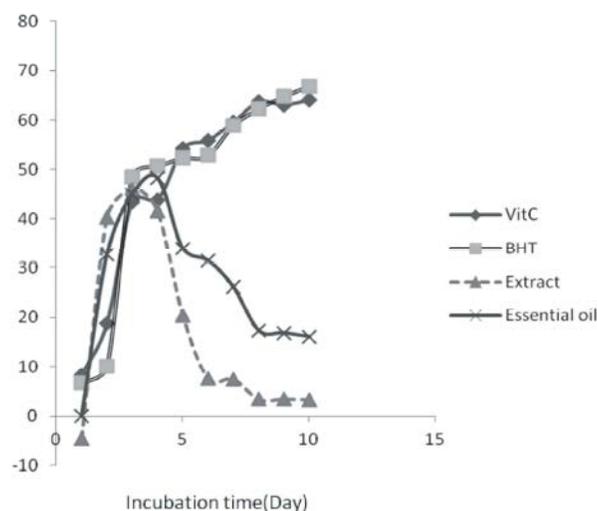


Fig. 1: Lipid peroxidation of Td extract and essential oil in comparison of BHT and Vit C as antioxidant standards.

Table 1: Antioxidant effects of *Thymus daenensis* by different method in comparison with antioxidant standards.

Sample	DPPH (IC ₅₀ , µg/ mL)	Nitric oxide scavenging ability % (200µg/mL)	FRAP	FTC	OH Scavenging (250 µg/mL)
TD Extract	194.24±0.021	74±0.0	10.16 ± 1.6	46.04 ± 0.24	60.21±0.27
TD Essential oil	> 3200 µg/mL	35±0.77		48.33 ± 0.12	54.71 ± 0.17
Galic acid	41±0.06				70.80±3.27
BHT	46.67± 1.19	42±0.79		66.86±0.15	72.47± 1.1
Quercetin	26.72±0.31				
Ascorbic acid				64.06±0.02	

Results are given as mean± SD values.

DISCUSSION

Geraniol is found as the main component of the collected *Td* Celak essential oil in this study. The results of several reports represent the existence of geraniol in the essential oil components of different *Thymus* species [9-11]. However, few reports indicate the rich amount of geraniol in *Td* [12]. Investigations have been revealed that geraniol is an effective plant-based substance. On the other hand, the replacement of geraniol with thymol in present study can be considered. These amounts of geraniol may be beneficial for clinical and industrial studies. It is a commercially monoterpene alcohol which it is widely applied in the flavoring industry [13,14]. As an essential oil component, it exhibits potent antimicrobial activity [15] and also is found to be effective as an antifungal agent [16]. Besides these effects, anticancer activities of geraniol have been evaluated and resulted in valuable outcomes. A new study represented that geraniol inhibits prostatic cancer growth and also increases the sensitivity of affected cells to chemotherapeutic agents [20]. Moreover the antitumor activity of geraniol has been investigated in pancreatic cancer and revealed that pancreatic tumor growth would be effectively inhibited when geraniol is given to the cell line [17]. Other studies presented the inhibitory effects of geraniol in human breast cancer [18], human hepatocellular carcinoma cell lines [19] and glycoprotein synthesis during malignant transformation [20]. Combination of geraniol and simvastatin indicated a synergistic effect on a human hepatocarcinoma cell (Hep G2). This combination also induces inhibition of cholesterol biosynthesis and proliferation of Hep G2 cell line [19].

Geraniol also exhibits anti-inflammatory effects on monocytic cell line [21] and suppresses the cellular inflammation in animal models [22]. Considering the rich amount of geraniol in mentioned *Thymus* and its valuable effects, inform us that this plant may be a good candidate for further clinical approaches.

In an attempt to evaluate the antioxidant activities of extract and essential oil of *Td*, the different methods such as, FRAP, DPPH, FTC, OH radical scavenging and NO scavenging are used.

In the present of antioxidant compound, purple color of DPPH solution is disappeared and colorless product is formed [23,24]. This method exhibits hydrogen donating effect of antioxidant compound [24].

Table 1 shows radical scavenging effects of *Td* extract and essential oil, in comparison with standards such as gallic acid, BHT and quercetin. Higher antioxidants have Lower IC₅₀ value. Between extract and essential oil of *Td*, extract has higher antioxidant activity (IC₅₀ = 194 ± 0.02 µg/ mL) but essential oil didn't show powerful activity in this method. The IC₅₀ of *Td* extract is lower than antioxidant standards BHT, gallic acid and quercetin ($p < 0.001$), but there is not any significant difference between IC₅₀ of antioxidant standards ($p > 0.05$). Regarding to the results of this study *Td* extract can be mentioned as natural antioxidant compound.

T. tosevii var. *tosevii* (Kicevo), *T. tosevii* var. *degenii* (Kitka), *T. tosevii* var. *longifrons* (Kitka), *T. tosevii* ssp. *substriatus* (Kavadarci), *T. longidens* var. *lanicaulis* (Sonje) [25] and *T. caramanicus* [26] are some of other *Thymus* species which introduced as natural resources of antioxidant in previous investigations.

In FRAP assay, antioxidants reduce the ferric-tripyridyl triazine (Fe³⁺-TPTZ) complex to blue ferrous (Fe²⁺-TPTZ) complex. This reduction progress at low pH and leads to change the color of complex at 593 nm [27]. In this test, standard curve was drawn between 1000-10000 µmol concentrations of FeSO₄. The results were expressed as µmol of FeSO₄ iron equivalents per g of sample. The results (Table 1) show the antioxidant potential of *Td* extract in FRAP assay is 10.16 ± 1.6 µmol/g of extract but *TD* essential oil is not active by this method.

Nitric oxide is an effective gas in both physiological and pathological conditions. In No scavenging assay, sodium nitroprusside is a source of free radicals and

products of nitrite and sulphanilamide reaction, coupled with naphthyl ethylene diamine and form chromophore. Antioxidant could prevent chromophore formation and absorbance reduction, thus absorbance of this chromophore is an indicator of NO scavenging activity [28].

The results of NO assay, demonstrate that NO scavenging of Td extract (74 ± 0) is more than Td essential oil ($34 \pm 0.77, p < 0.001$) and BHT ($42 \pm 0.79, p < 0.001$, Table 1).

In Ferric thiocyanate (FTC) assay, primary products of lipid oxidation are evaluated. These products are peroxides, which form during the early stages of oxidation process [10]. Figure 1 shows, extract and essential oil of Td have moderate antioxidant activities in comparison to BHT and ascorbic acid. In FTC assay there is a significant difference ($p < 0.05$) between extract and essential oil of Td, but this difference is ($p < 0.001$) in comparison with antioxidant standards like BHT and ascorbic acid.

In OH scavenging, between Td extract and essential oil a significant difference is observed, ($p < 0.05$, Table 1). Hydroxyl radical scavenging of Td extract (60.2 ± 0.27) and Td essential oil (54.7 ± 0.17) are less than gallic acid and BHT (70.8 ± 3.3) and BHT (72.24 ± 3.7 , Table 1, $p < 0.001$).

Lipid peroxidation inhibition by vit C and BHT increase until twelve days, but this inhibition was stopped by BHT and vit C in fifth days. Anyway the inhibition of lipid peroxidation by Td extract (46.06 ± 0.24) and Td essential oil (48.3 ± 0.12) are less than antioxidant standards BHT (66.86 ± 0.15) and ascorbic acid (64.06 ± 0.2).

In conclusion, other research is reported that geraniol compound inhibits lipid peroxidation, decreases No release and induces SOD, GSH enzymes activities [28]. Thus in this research, the antioxidant properties of Td may be correlated to its geraniol contents.

ACKNOWLEDGEMENT

The current research was supported by Shiraz University of Medical Sciences Grant.

Author Disclosure Statement: No competing financial interests exist.

REFERENCES

1. Rechinger, K., 1982. Flora Iranica, Austria, Akademische druck-u. Verlagsanstalt.

2. Mahmoudi, M., K. Morteza-Semnani and E. Mojra, 2008. Anti-inflammatory and antinociceptive activity of Thymus pubescens extract, Fitoterapia, 79(5): 361-365.
3. Stahl-Biskup, E. and F. Saez, 2002. Thyme (The Genus Thymus), pp: 75-73. Taylor and Francis, New York.
4. Masada, Y., 1976. Analysis of essential oils by gas chromatography and mass spectrometry, Medium: X; Size: Pages: pp: 340.
5. Adams, R.P., 1995. Identification of essential oil components by gas chromatography–quadropole mass spectroscopy. Allured Publishing, Illinois.
6. Moein, S., M.R. Moein, S. Khaghani and B. Farzami, 2010. Antioxidant properties of Zhumeria majdae Rech.f. & Wendelbo, Journal of Pharmacy Research, 3(1): 34-38.
7. Lee, S.H., J.B. Lee, K.W. Lee and Y.J. Jeon, 2010. Antioxidant properties of tidal pool microalgae, Halochlorococcum porphyrae and Oltamanniellopsis unicellularis from Jeju Island, Korea, Algae, 25(1): 45-56.
8. Olabinri, B.M., O.O. Odedire, M.T. Olaleye, A.S. Adekunle, L.O. Ehigie and P.F. Olabinri, 2010. *In vitro* Evaluation of Hydroxyl and Nitric Oxide Radical Scavenging Activities of Artemether, Research Journal of Biological Sciences, 5(1): 102-105.
9. Mockute, D. and G. Bernotiene, 1999. The main citral-geraniol and carvacrol chemotypes of the essential oil of thymus pulegioides L. growing wild in vilnius district (Lithuania), J. Agric. Food Chem, 47(9): 3787-3790.
10. Radonic Ani, Mastic and Josip, 2008. Essential Oil and Glycosidically Bound Volatiles of Thymus pulegioides L. growing Wild in Croatia, pp: 8 Croatian Chemical Society, Zagreb.
11. De Martino, L., M. Bruno, C. Formisano, V. De Feo, F. Napolitano, S. Rosselli and F. Senatore, 2009. Chemical composition and antimicrobial activity of the essential oils from two species of Thymus growing wild in southern Italy, Molecules, 14(11): 4614-4624.
12. Bahreininejad, B., Mirza, Mehdi, Arzani and Ahmad, 2010. Essential Oil Variation in Thymus daenensis subsp. daenensis Cleak Populations, pp: 4 Allured, Carol Stream, IL, ETATS-UNIS.

13. Lapczynski, A., S.P. Bhatia, R.J. Foxenberg, C.S. Letizia and A.M. Api, 2008. Fragrance material review on geraniol, Food and Chemical Toxicology, 46(11): 160-170.
14. Chen, W. and A.M. Viljoen, 2010. Geraniol-A review of a commercially important fragrance material, South African Journal of Botany, 76(4): 643-651.
15. Aridogan, B.C., H. Baydar, S. Kaya, M. Demirci, D. Ozbasar and E. Mumcu, 2002. Antimicrobial activity and chemical composition of some essential oils, Arch Pharm Res., 25(6): 860-864.
16. Bard, M., M.R. Albrecht, N. Gupta, C.J. Guynn and W. Stillwell, 1988. Geraniol interferes with membrane functions in strains of Candida and Saccharomyces, Lipids, 23(6): 534-538.
17. Kim, S.S., O.J. Oh, H.Y. Min, E.J. Park, Y. Kim, H.J. Park, Y. Nam Han and S.K. Lee, 2003. Eugenol suppresses cyclooxygenase-2 expression in lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells, Life Sci., 73(3): 337-348.
18. Duncan, R.E., D. Lau, A. El-Sohemy and M.C. Archer, 2004. Geraniol and beta-ionone inhibit proliferation, cell cycle progression and cyclin-dependent kinase 2 activity in MCF-7 breast cancer cells independent of effects on HMG-CoA reductase activity, Biochem Pharmacol, 68(9): 1739-1747.
19. Polo, M.P., R. Crespo and M.G. De Bravo, 2011. Geraniol and simvastatin show a synergistic effect on a human hepatocarcinoma cell line, Cell Biochemistry and Function, 29(6): 452-458.
20. Vinothkumar, V., S. Manoharan, D. Palanimuthu, D. Rajasekaran, R. Srinivasan and A.W. S, 2011. Geraniol protects cell surface glycoconjugates abnormalities during 7, 12-dimethylbenz (a) anthracene induced oral carcinogenesis Journal of Cell and Tissue Research, 11(2): 2759-2764
21. Marcuzzi, A.S. Crovella and A. Pontillo, 2011. Geraniol rescues inflammation in cellular and animal models of mevalonate kinase deficiency, In Vivo, 25(1): 87-92.
22. Maruyama, N., Y. Sekimoto, H. Ishibashi, S. Inouye, H. Oshima, H. Yamaguchi and S. Abe, 2005. Suppression of neutrophil accumulation in mice by cutaneous application of geranium essential oil, J Inflamm (Lond), 2(1): 1.
23. Natrah, F., F. Yusoff, M. Shariff, F. Abas and N. Mariana, 2007. Screening of Malaysian indigenous microalgae for antioxidant properties and nutritional value, Journal of Applied Phycology, 19(6): 711-718.
24. Amiri, H., 2012. Essential Oils Composition and Antioxidant Properties of Three Thymus Species, Evidence-Based Complementary and Alternative Medicine, pp: 1-8.
25. Sokmen, A., M. Gulluce, H. Askin Akpulat, D. Daferera, B. Tepe, M. Polissiou, M. Sokmen and F. Sahin, 2004. The *in vitro* antimicrobial and antioxidant activities of the essential oils and methanol extracts of endemic Thymus spathulifolius, Food Control, 15(8): 627-634.
26. Safaei-Ghomi, J., A.H. Ebrahimabadi, Z. Djafari-Bidgoli and H. Batooli, 2009. GC/MS analysis and in vitro antioxidant activity of essential oil and methanol extracts of *Thymus caramanicus* Jalas and its main constituent carvacrol, Food Chemistry, 115(4): 1524-1528.
27. Benzie, I.F.F. and J.J. Strain, 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay, Analytical Biochemistry, 239(1): 70-76.
28. Acharyya, S., G.K. Dash, S. Mondal and S.K. Dash, 2010. Antioxidant and antimicrobial study of Spondias M angifera wild root International Journal of Pharmacy and Pharmaceutical Sciences, pp: 2.