Trad. Med. J., September 2014
Vol. 19(3), p 142-148
ISSN : 1410-5918

Submitted : 10-01-2014
Revised : 10-12-2014
Accepted : 24-12-2014

ISOLATION AND IDENTIFICATION OF FREE RADICALS SCAVENGER FROM *Daucus carota* L LEAVES

Sri Sudewi[1], Subagus Wahyuono[2], Puji Astuti[2*]
[1]Dept of Pharmacy, University Sam Ratulangi, Manado, Northern Sulawesi, Indonesia
[2]Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara Yogyakarta, Indonesia

ABSTRACT

The presence of free radicals produced inside the cells is thought to cause a range of diseases such as degenerative diseases. Antioxidant properties of carrot root (Daucus carota L.) have been reported elsewhere to inhibit the formation of these free radicals, but the compounds responsible for antioxidant activity inside its leaves have not yet determined. In Indonesia, Daucus carota L. leaves are regarded as waste product after harvesting the root. This study was conducted to isolate and identify the compound having antioxidant activity from the leaves of D.carota. n-Hexane, ethyl acetate, and methanol extracts were examined for free radical scavenger activity by using DPPH method. The n-hexane extract showing free radical scavenger activity was solid-solvent partitioned, fractionated using vacuum liquid chromatography and tested for free radical scavenger activities. The active compound was isolated by using preparative thin layer chromatography and its purity was determined by TLC with three mobile phase systems and two dimensional TLC. Based on spectroscopy UV-Vis, IR as well as 1H-NMR (CDC13) data, the active compound was suggested to be xanthophyll derivate, lutein. The compound at concentration of 0.616, 1.025, and 2.05 ppm had antioxidant activities of 0.94 ± 0.05; 18.53 ± 0.15; and 49.07 ± 0.86, respectively.
Key words: Leaves, D.carota, antioxidant, free radical

INTRODUCTION

There is a huge amount of evidence showing the contribution of free radicals in mediating the development of human diseases such as cancer, degenerative diseases and neuronal diseases (Halliwell *et al.,*1992, Thomas & Kalyanaraman, 1997, Waley-Connell *et al.,* 2011). Free radicals such as hydrogen peroxide (H2O2); superoxide (Oz^-); and the hydroxyl radical (•OH). are atom or atomic groups belongs to one of unpaired electron and therefore are highly reactive molecules (Fessenden and Fessenden, 1995). Free radicals were formed naturally and continued present in human body. If the formation exceeds the antioxidant capacity of an organism, it will induce a variety of pathophysiological processes lead to various diseases. Chain reaction will stop when the free radicals is scavenged by molecules namely antioxidant (Halliwell and Gutteridge, 1985). Antioxidants have been reported to inhibit the formation of free radicals through removing their intermediates. A number of natural antioxidants have been explored and developed to protect the body against these harmful molecules. Rutin and phenolic compounds isolated from Trans-Himalayan plants have been reported to have antioxidant property (Yang et al, 2008; Kogje et al., 2010). Vitamin C, tocoferol, phenol, and carotenoid isolated from plants scavenged the free radicals and broke the chain reactions (Fang et al., 2002).

Carrot (*Daucus Carota* L.) is plant regarded as one top 10 level of vegetables having nutrition value compared to other 39 fruits and vegetables (Sun *et al.,* 2009). Carrot contains carotenoid (β-caroten, α-caroten, γ- caroten, licopen, lutein, kriptoxantin) dan phenolic compounds (phenil propanoid, eugenol, miricetin) (Ross, 2005), flavonoid (quercetin, rutin, luteolin) (Muralidharan *et al.,* 2008), vitamin C (Kumar et al., 2004) and polyacetylenes (Baranska and Schulz, 2005, Purup *et al.,* 2009). Flavonoid, phenolic compounds and vitamin C are reported to have antioxidant since they are able to donate hydrogen/electron to stabilize radical species (Lopez-lazaro, 2009) whilst carotenoid can bind singlet oxygen or deactivate oxygen radical.

Carrot consists of roots and leaves. Its roots are usually the one which are used as food, vegetables and source of drink. There have been a lot of studies conducted about the roots (Smallwood, et al., 1999, Purup et al., 2009, Roman *et al.,* 2011) but less about the leaves. In Indonesia the leaves are only used by farmers as source of fertilizer and animal food. There are only few studies conducted to determine the activity or nutrition values of this material. Recent study

Corresponding author : Puji Astuti
E-mail: puji_astuti@ugm.ac.id

reported that carrot leaves have antioxidant capacity and used as snack (Limsangouan, et al 2010). However, the biological active responsible for this antioxidant activity has not yet determined. Based on the characteristic of plant chemical compound distribution, chemical components of plants can translocalise from one part to the other, the term normally called translocalisation processes. These chemical components could move from leaves to the roots, fruits, flowers, stem, and young leaves (Kour, 2006). The distribution and quantity of the chemical components varies between parts of the plants, depend on its function. Since carrot's roots have been reported to have compounds having antioxidant properties, it is possible that its leaves also contain components having the same properties. This study is conducted to isolate and identify antioxidant compounds present in the carrot's leaves.

METHODOLOGY

Carrot's leaves were collected from Tawangmangu, Karanganyar, Central Java on January 6, 2011. The sample was authenticated at Faculty of Pharmacy, Universitas Gadjah Mada Yogyakarta and voucher specimen no 477 was deposited at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. 1,1-Diphenyl-2-picryl-hydrazyl (DPPH) was purchased from Sigma–Aldrich (USA). The solvents and other chemicals were of analytical grade except those used for vacuum liquid chromatography.

Procedure

Extraction and isolation

Carrot's leaves were cleaned and dried in the oven at 50^{o}C for 2x24 hour and then powdered. The powder was extracted gradually using n-hexane, ethyl acetate and methanol to obtain crude extracts of n-hexane, ethyl acetate and methanol, respectively. These extracts were tested qualitatively for free radical scavenger activity based on the method described in Masoko and Ellof (2007). The presence of yellow spots against purple background on TLC indicating the presence of antioxidant compounds. The positive extract was partitioned using solid-solvent extraction and further fractionated using vacuum liquid chromatography using silica gel 60 F254 as stationary phase and in increasing proportions (10%) of n-hexane: ethyl acetate as mobile phases. The fractions were separated by TLC and those having similar profiles were combined and tested for antioxidant properties. The positive fractions were separated by preparative-TLC and the antioxidant activity of the isolated compounds were tested using qualitative metal ion-chelating assay as described in Rohman et al., (2010). The purity of the isolate was determined by two dimensional TLC and TLC with various compositions of mobile phases.

Structure elucidation

The structure of the active compound was elucidated based on UV-Vis spectrophotometric (Hitachi U-2800), Infra Red (PERKIN ELMER FTIR 100) and 1H-NMR (JEOL JNM ECA DELTA2 with frequency 500 MHz) data.

Antioxidant activity

Antioxidant assay used for fractionation and isolation of antioxidant compounds were determined qualitatively according to Masoko and Ellof 2007. Briefly, the extracts were separated by TLC, the plates are air-dried and the chromatograms were sprayed with 0.2% 2,2, diphenyl-2-picryl-hydrazyl (DPPH) in methanol. The presence of yellow spots against purple background indicating the presence of antioxidant compounds. To determine antioxidant activity of the isolate quantitatively, metal-ion-chelating assay was used according to Rohman et al, (2010). The samples (mL) were added to 0,05 mL 2mM $FeCl_2$. After adding 0,2 mL 5 mM ferrozin, the samples were mixed vigorously and let stand at room temperature for 10 minutes. After equilibrium, the absorbance was measured using spectrophotometer at wave length 562 nm. The experiment was conducted triplicate.

$$\text{Percentage (\%) of chelating inhibition} = \frac{(A_0-A_1)}{A_0} \times 100$$

Where : A_0 was the absorbance negative control and A_1 was absorbance of the samples. Ethylene diamintetraacetate (EDTA) was used as positive control

RESULTS AND DISCUSSION

TLC-bioautography guided isolation of antioxidant compound

Based on identification of antioxidant compounds using 0.2% DPPH solution sprayed on to TLC chromatogram, the strongest free radical scavenging activity was shown by n-hexane extract (Figure 1). The DPPH method was used based on the ability of antioxidant compounds to neutralize the generation of free radicals. Molecules which are able to donate electron or hydrogen within the solution will change the color of DPPH from purple into yellow spot.

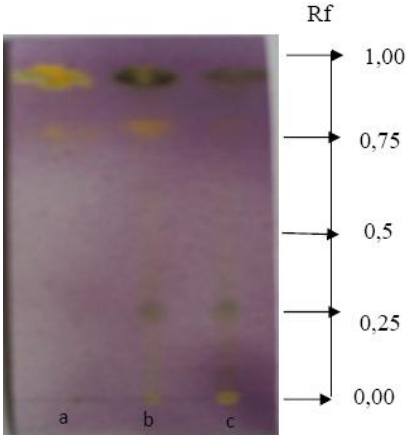

Figure 1. TLC plate sprayed with 0.2% DPPH solution in methanol and visualized under visible light. (a) n-hexane, (b) ethyl acetate, (c) methanol extracts. Stationary phase: silica gel GF 254, mobile phase chloroform: ethyl acetate (3:1 v/v). The presence of yellow spots against the purple background indicating the presence of antioxidant compounds.

The n-hexane extract was then partitioned using methanol to obtain methanol-soluble and non soluble fractions. The positive fraction (methanol-non soluble fraction) was further separated by vacuum liquid chromatography (VLC). The efficiency of separation and the presence of antioxidant compounds were determined by TLC sprayed with 0.2% DPPH in methanol. From twelve fractions, those having similar TLC profiles (fraction VI-XII) were combined to obtain six fractions (Figure 2).

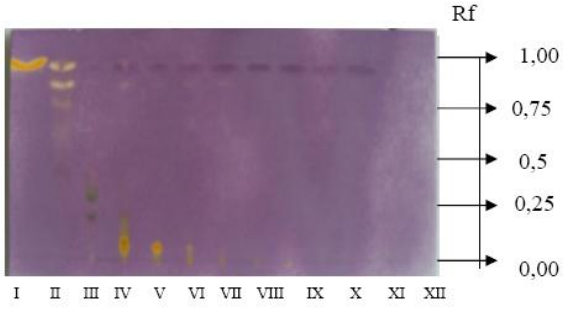

Figure 2. Vacuum liquid chromatography profiles of methanol non-soluble fraction of n-hexane extract. Stationary phase: silica gel 60 F254, mobile phase n-hexane: ethyl acetate with 10% increased level of ethyl acetate. The final wash was performed using methanol. Fraction VI to XII were combined and designated as fraction VI.

It has been reported that volatile oil, which commonly present in non-polar extract, has antioxidant activity (Mansour et al., 2001; Barra et al., 2007; Kapoor et al., 2009), therefore the volatile oil obtained by steam distillation of fresh leaves was tested for free radical scavenging activity by TLC sprayed with 0.2% DPPH solution. However, the volatile oil turned out showing negative result compared to that of fraction I (Figure 3). These data suggested that the antioxidant activity of n-hexane extract was not due to the presence of volatile oil.

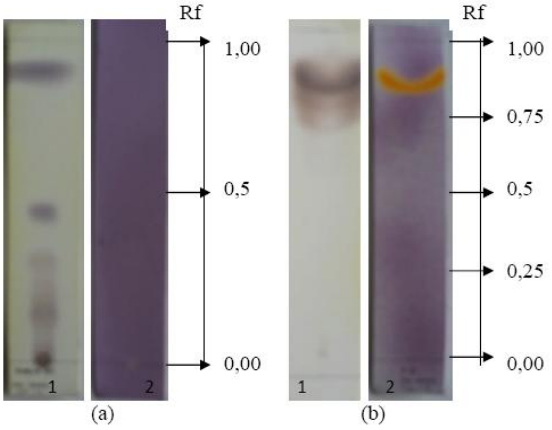

Figure 3. Volatile oil obtained from carrot's leaves by steam distillation did not have antioxidant activity. The volatile oil (a) and fraction I obtained from VLC (b) were separated by TLC and sprayed with cerium (IV) sulphate (1) and 0.2% DPPH solution. The presence of yellow spot indicating the presence of antioxidant compounds.

In this study, although fraction I showed highest intensity, fraction V was of interest to be isolated and characterized due to the abundant of sample and the higher rate of color change compared to others. The antioxidant compound present in fraction V was then separated by preparative thin layer chromatography (TLC) and the purity of this compound was determined by two dimensional TLC and TLC with various mobile phases. The present of single spot with these two methods indicated the level of purity of the isolate (Figure 4).

Antioxidant activity

The antioxidant capacity of this isolate was tested using metal ion-chelating method as described in Rohman *et al,* (2010). Based on this method, antioxidant activity was determined by the ability of a compound to abrogate ferrozine-Fe^{2+} complex, reducing the intensity of red color developed from this complex. The isolate at the concentration of 0.616, 1.025 and 2.05 ppm have

metal ion-chelating activity of 0.94 ± 0.05, 18.53 ± 0.15 and 49.07 ± 0.86 % respectively. Increasing concentration of isolate showed increased activity. EDTA which was used as positive control in this experiment has IC_{50} of 18.19 ppm. It is suggested that the isolate has stronger antioxidant activity compared to that of control.

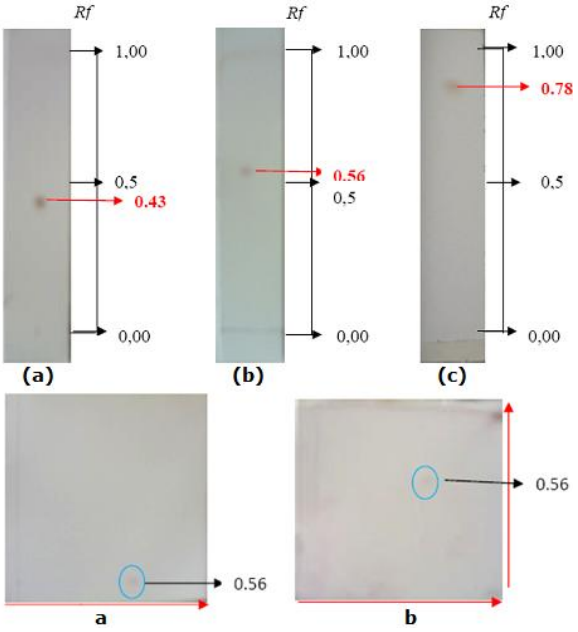

Figure 4. The purity of isolate from fraction V as determined by TLC with various mobile phases, n-hexane:etyl acetate (2:1 v/v) (a), chloroform: ethyl acetate (9:1 v/v) (b), chloroform:ethyl acetate (1:1 v/v) (c) and two dimensional TLC with chloroform: ethyl acetate (9:1 v/v) as mobile phases for first (d) and second (e) elution. Stationary phase: silica gel 60 GF254.

Structure elucidation

Due to its antioxidant capacity, it is interesting to characterize further the structure of this active compound. Preliminary identification of the active compound showed the presence of terpenoids as indicated by green color spot with Lieberman-Buchard detection reagent (Figure 5). UV-Vis spectrophotometric data showed λ_{max} at 432, 455, and 485 nm indicating the presence highly chromophoric bonds. Based on this data, the isolated compound (Figure 6) was predicted to have many conjugated double bonds such as carotenoids type of compounds (Harborne, 1987).

Absorption in the area of 3445 cm^{-1} in the IR spectrum indicated the present of OH stretch, whilst at 2927 cm^{-1} and low absorption at 1425 cm^{-1} indicated the presence of C-H stretch and (-CH2-), respectively (Silverstein *et al.,* 1991). Strong absorption was seen in the area of 1630 cm^{-1} (C=C-C=C, stretch, symmetry) (Figure 7).

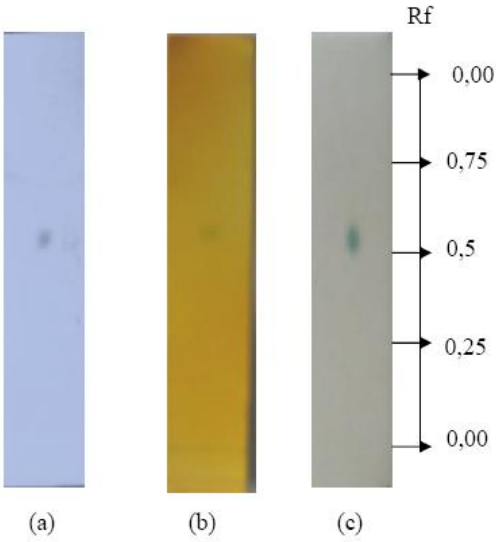

Figure 5. TLC profile of active compound indicated the presence of terpenoid. The isolate was eluted using silica gel 60 GF 254 as stationary phase and chloroform: ethylacetate (9:1 v/v) as mobile phase and spayed with $AlCl_3$ (a), Dragendoff (b) and Liberman-Burchard (c).

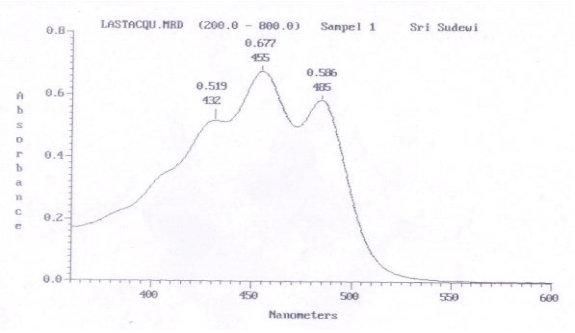

Figure 6. Ultraviolet spectrum ($CHCl_3$) of the isolated compound

According to Bruneton (1999) carotenoid can be categorized into two groups based on its structure elements, namely carotene which contains -C and -H and xanthophyll, which consists of atomics C, H and O. The presence of OH group suggested that the isolated compound was a xanthophyll derivate, possibly identified as Lutein. Further characterization of this compound by ^{1}HNMR showed chemical shift of 0.8 – 1.1 ppm (singlet) of geminal methyl protons and 1.63-1.73 ppm indicated the present of unsaturated methyl proton such as allylic methyl groups. The more down field methylene proton was bound to an alkene with conjugated double bound (1.91 – 1.96

ppm) and a methyne proton (-CH=) conjugated to a double bound 5.43 – 6.64 ppm were also observed (Hwang *et al.,* 2005) (Figure 8, Tabel I).

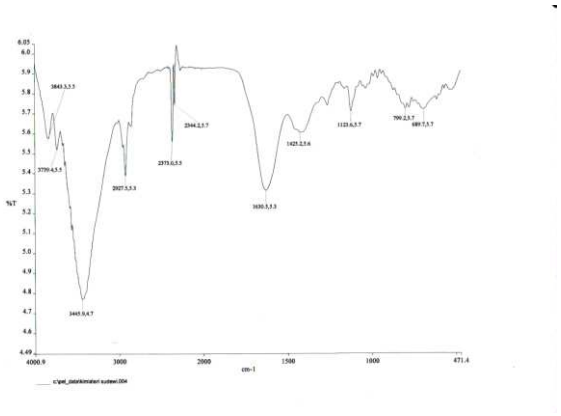

Figure 7. Infrared spectrum (KBr) of the isolated compound

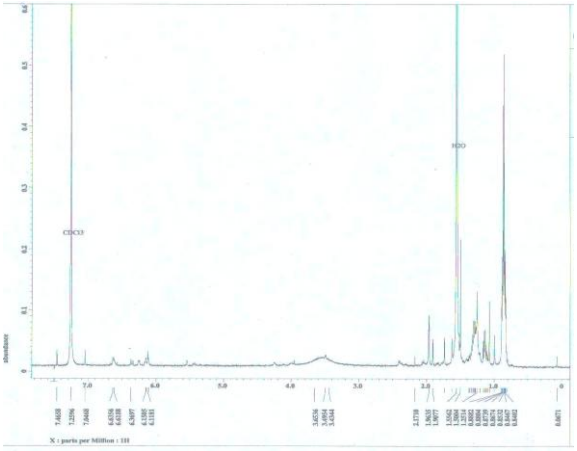

Figure 8. ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of the isolated compound

Based on the spectrum information, the antioxidant compound isolated from the carrot leaves was suggested to be lutein (Figure 9).

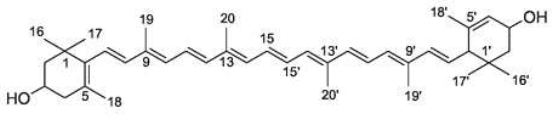

Figure 9. Lutein

Table I. 1HNMR data of isolate compared to lutein (Hwang *et al.,* 2005)

No H	Isolat (δ) (ppm)	Lutein (Hwang dkk., 2005) (δ) (ppm)
1	-	-
2	**1,32 (Hax–2, m)** 1,83 (Heq–2, m)	**1,48 (Hax-2, m, 1H)** 1,84 (Heq-2, m, 1H)
3	3,9 (H-3, m)	4,00 (H-3, m, 1H)
4	2,05 (Hax–4, dd) 2,35 (Heq–4, dd)	2,05 (Hax-4, dd, J=5,0, 18,1 Hz, 1H) 2,38 (Heq-4, dd, J=5,0, 18,1 Hz, 1H)
5	-	-
6	-	-
7	6,10 (H-7, d)	6,09 (H-7, d, J=16,2 Hz, 1H)
8	6,14 (H-8, d)	6,14 (H-8, d, J=16 Hz, 1H)
9	-	-
10	6,11 (H-10, d)	6,13 (H-10, d, J=12 Hz, 1H)
11	6,63 (H-11 dan 11', m)	6,63 (H-11 dan 11', m, 2H)
12	6,36 (H-12, d)	6,36 (H-12, d, J=14,9 Hz, 1H)
13	-	-
14	6,25 (H-14 dan 14', m)	6,25 (H-14 dan 14', m, 2H)
15	6,64 (H-15 dan 15', m)	6,64 (H-15 dan 15', m, 2H)
16	1,09 (H-16 dan 17, s)	1,07 (H-16 dan 17, s, 6H)
17		
18	1,73 (H–18, s, 3H)	1,73 (H-18, s, 3H)
19	1,96 (H–19, 20, 20', s, 9H)	1,96 (H-19, 20, 20', s, 9H),
20		
1'	-	-
2'	**1,14 (Hax–2', dd)** 1,84 (Heq–2', dd)	**1,38 (Hax-2', dd, J=5,3, 13,9Hz, 1H),** 1,83 (Heq-2', dd, J=6,2, 13,2 Hz, 1H)
3'	4,25 (H-3', s)	4,25 (H-3', s, 1H)
4'	5,5 (H-4', s)	5,54 (H-4', s, 1H)
5'	-	-
6'	2,4 (H-6', d)	2,39 (H-6', d, J=9,2 Hz)
7'	5,4 (H-7', dd)	5,43 (H-7', dd, J=9,2, 15,5 Hz, 1H)
8'	6,16 (H-8', d)	6,16 (H-8', d, J=15,5 Hz, 1H)
9'	-	-
10'	6,15 (H-10', d)	6,15 (H-10', d, J=11,5 Hz, 1H)
11'	6,63 (H-11 dan 11', m)	6,63 (H-11 dan 11', m, 2H)
12'	6,34 (H-12', d)	6,34 (H-12', d, J=14,9 Hz, 1H)
13'	-	-
14'	6,25 (H-14 dan 14', m)	6,25 (H-14 dan 14', m, 2H)
15'	6,64 (H-15 dan 15', m)	6,64 (H-15 dan 15', m, 2H)
16'	0,85 (H-16', s)	0,85 (H-16', s, 3H)
17'	0,99 (H-17', s)	1,00 (H-17',s, 3H)
18'	1,62 (H-18', s, 3H)	1,63 (H-18', s, 3H)
19'	1,9 (H–19', s, 3H)	1,91 (H-19', s, 3H)
20'	1,96 (H-19, 20, 20', s)	1,96 (H-19, 20, 20', s, 9H)

Lutein is well known antioxidant and anti free radical scavenger, widely used in cosmetics and neutraceutical industry. This compound was found to be able to scavenge free radicals (Gowda et al., 2010), inhibit hydrogen peroxide-NFκB and IL-8 expression in gastric epithelial AGS cells (Kim *et al* 2011), and inhibit the growth of mouse mammary tumor (Chew *et la.,* 2003). The presence of lutein in the carrot's leaves suggested the important of this part of plant as source of antioxidant. This compound was found to be abundant compared to the root (Figure 10), and utilization of waste carrot leaves for neutraceuticals products therefore is promising.

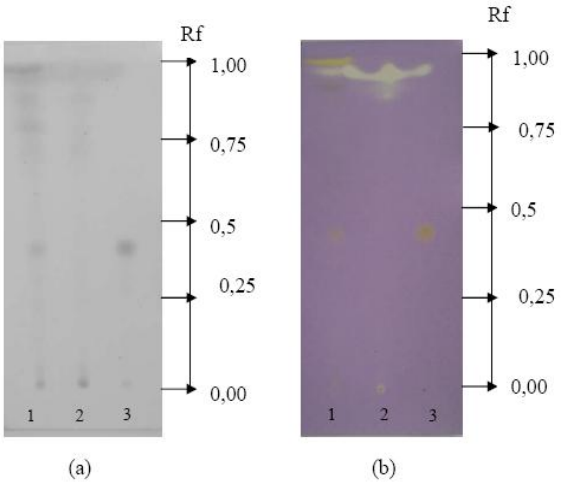

Figure 10. TLC profiles n-hexane extract of *D. carota* leaves (1), root (2) and the isolate (3) as determined by cerium (IV) sulfate detection reagent (a) and 0.2% DPPH (b). The same amount of extract was loaded into the plate. Silica gel 60 GF254 as stationary phase and n-hexane: ethyl acetate (2;1 v/v) as mobile phase.

CONCLUSION

Carot (*D.carota*) leaves contains antioxidant compounds with lutein suggested to be one of the active isolate. This compound was found to be abundant compared to that in the root. This isolate had metal ion-chelating inhibition activity at concentration dependent manner and had stronger effect compared to EDTA control.

REFERENCES

Baranska, M, and Schulz, H., 2005, Spatial tissue distribution of polyacetylenes in carrot root, *Analyst*,130(6):855-9

Barra, A., Coroneo, V., Dessi, S., Cabras, P., Angioni, A., 2007, Characterization of the volatile constituents in the essential oil of Pistacia lentiscus L. from different origins and its antifungal and antioxidant activity, *J Agric Food Chem.,* 55(17):7093-8.

Bruneton, J., 1999, *Pharmacognosy, Phytochemistry, Medicinal Plants*, 2nd ed, translated by Halton, C.K., Lavoisier Publishing Inc, Intercept Ltd, Technique and Documentation, 769.

Chew, B.P., Brown, C.M., Park, J.S., and Mixter, P.F., 2003, Dietary lutein inhibits mouse mammary tumor growth by regulating angiogenesis and apoptosis, *Anticancer Res*, 23, 3333-3339.

Fang, Y.Z., Yang, S., and Wu, G., 2002, Free radicals, antioxidant and nutrition, *Nutrition*, 18, 872-879.

Fessenden, R.J., dan Fessenden, J.S., 1995,*Organic Chemistry*, Willard Grant Press, Boston, (Indonesian), translated by Pudjaatmaka A.H., third ed., Erlangga, Jakarta

Gowda, S.S.T., R. Dinesha, R. Harsha, and Srinivas, L., 2010, Free radical scavenging activity of lutein – isolated from Methi leaves (*Trigonella foenum graecum*), *International Journal of Pharmacy and Pharmaceutical Sciences*, 2, 113-117.

Halliwell, B., and Gutteridge, J.M.C.,1985, *Free radicals in biology and medicine*, Oxford: Clorendon Press

Halliwell, B., Gutteridge, J.M.C., and Cross, C.E., 1992, Free radicals, antioxidants and human disease: where are we now?, *Journal of Laboratory and Clinical Medicine,* 119, 598-620.

Harborne, J.B., 1987. *Phytochemical Methods*. Chapman and Hall, London.

Hwang, S.W., Lee, J., Kwon, H.S., Lee, K.D., Nam, S.H., Park, K.H., and Yang, M.S., 2005, Comparison of tyronase inhibitory effect of the natural antioxidants from *Cedrela sinensis*, *Agric. Chem. Biotechnol*, 48 (3), 144-147.

Kapoor, I.P., Singh, B., Singh, G., De Heluani, C.S., De Lampasona, M.P., Catalan, C.A., 2009, Chemistry and in vitro antioxidant activity of volatile oil and oleoresins of black pepper (*Piper nigrum*), *J Agric Food Chem*., 57(12):5358-64.

Kim ,Y., Seo, J.H., Kim, H., 2011, β-Carotene and lutein inhibit hydrogen peroxide-induced activation of NF-κB and IL-8 expression in gastric epithelial AGS cells, *J Nutr Sci Vitaminol* (Tokyo), 57(3):216-23.

Kogje, K.K., Jagdale, V.K., Dudhe, S.S., Phanikumar, G., and Badere, R.S., 2010, Antioxidant property and Phenolic Compounds of few Important Plants from Trans-Himalayan Regions of North India, *Journal of Herbal Medicine and Toxicology,* 4 (2) 145-151.

Kour, H., 2006, *Encyclopaedia of Plant Physiology*, Vol 2, Anmol Publications Pvt.Ltd., New Delhi, India.

Kumar, S., Dhingra, A., and Daniell, H., 2004, Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots and leaves confers enhanced salt tolerance, *Plant Physiology*, 136, 2843-2854.

Limsangouan, N., Takenaka, M., Sotome, I., Nanayama, K., Charunuch, C., and Isobe, S.,2010, Functional Properties of Cereal and Legume Based Extruded Snack Foods Fortified with By-Products from Herbs and Vegetables. *Kasetsart J. (Nat. Sci.),* 44, 271 – 279

Lopez-Lazaro, M., 2009, Distribution and biological activities of the flavonoid luteolin, *Mini Reviews in Medicinal Chemistry,* 9(1); 31-59.

Mansou,r M.A., Ginawi, O.T., El-Hadiyah, T., El-Khatib, A.S., Al-Shabanah, O.A., Al-Sawaf, H.A., 2001, Effects of volatile oil constituents of Nigella sativa on carbon tetrachloride-induced hepatotoxicity in mice: evidence for antioxidant effects of thymoquinone, *Res Commun Mol Pathol Pharmacol.,* 110(3-4):239-51.

Masoko, P and Eloff, J.N., 2007, Screening of twenty-four South African Combretum and Six Terminalia Species (Combretaceae) for Antioxidant Activities, *Afr. J. Trad. CAM,* 4 (2): 231 - 239

Muralidharan, P., Balamurugan, G., and Kumar, P., 2008, Inotropic and cardioprotective effects of *Daucus carota* Linn. on isoproterenol-induced myocardial infarction. *Bangladesh Journal of Pharmacology*, 3, 74-79.

Purup, S., Larsen, E., Christensen, L.P., 2009, Differential effects of falcarinol and related aliphatic C(17)-polyacetylenes on intestinal cell proliferation, *J Agric Food Chem*., 57(18):8290-6.

Rohman A., Riyanto, S., Yuniarti, N., Saputra, W.R., Utami, R., and Mulatsih, W., 2010, Antioxidant activity, total phenolic, total flavonoid of extracts and fractions of red fruit (*Pandanus conoidens* Lam), *Int.Food.Res.J*.,17, 97-106.

Roman, M., Dobrowolski, J.C., Baranska, M., Baranski, R., Spectroscopic studies on bioactive polyacetylenes and other plant components in wild carrot root., 2011, *J Nat Prod*., 74(8):1757-63.

Ross, I.A., 2005, *Medicinal Plants of the World volume 3 Chemical Constituents, Traditional and Modern Uses*, Humana Press Inc, New Jersey.

Silverstein, R.M., Bassler, G.C., and Morril, T.C., 1991, *Spectrometric Identification of Organic Compound,* Fifth Edition, John Wiley and Sons, New York.

Smallwood, M., Worrall, D., Byass, L., Elias, L., Ashford, D., Doucet, C.J., Holt, C., Telford, J., Lillford, P., Bowles, D.J., 1999, Isolation and characterization of a novel antifreeze protein from carrot (*Daucus carota*)., *Biochem J*., 340 (Pt 2):385-91.

Sun, T., Simon, P.W., and Tanumihardjo, S.A., 2009, Antioxidant phytochemical and antioxidant capacity of biofortified carrots (*Daucus carota* L.) of various colors. *Journal of Agriculture and Food Chemistry*, 57, 4142–4147

Thomas, C. E., & Kalyanaraman, B.,1997, *Oxygen radicals and the diseases process*, The Netherlands: Hardwood Academic Publishers.

Whaley-Connell, A., McCullough, P.A., Sowers, J.R., 2011, The role of oxidative stress in the metabolic syndrome, *Rev Cardiovasc Med.*, 12(1):21-9.

Yang, J., Guo, J., Yuan, J., 2008, In vitro antioxidant properties of rutin, *LWT-Food Science and Technology*, 41, 1060–106