ISSN : 1225-9918
Journal of Life Science **2011** Vol. 21. No. 6. 775~782 DOI : 10.5352/JLS.2011.21.6.775

Antioxidant Capacity and Quinone Reductase Activity of Methanol Extracts and Fractions from Papaya Seed

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Received March 31, 2011 / Accepted April 24, 2011

In this study, the antioxidant activity of methanol extracts and fractions from papaya seed were investigated *in vitro*. Total polyphenol contents of methanol extracts and fractions from papaya seed varied from 17.74 to 125.99 μg/mg and total flavonoid contents varied from 1.60 to 32.69 μg/mg. Contents of polyphenol and flavonoid in ethyl acetate (EtOAc) fraction was found to be extremely high (compared with the other fractions examined). Radical-scavenging activities of methanol extracts and fractions were examined using α,α-diphenyl-β-picrylhydrazyl (DPPH) radicals, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide assay. As a result, ethyl acetate fraction of papaya seed showed the highest radical-scavenging activity in various antioxidant systems. The EtOAc fraction from papaya seed induced QR activity in concentrations of 12.5 to 50 μg/ml with a maximum of a 3.3-fold induction at 50 μg/ml of fraction. Therefore, the most effective QR inducer among these fractions can be said to reside in the EtOAc fraction, indicating that strong constituents responsible for QR induction potency in the papaya seed extract are largely contained in the EtOAc fraction.

Key words : Papaya, seed, antioxidant, quinone reductase, Hepa1c1c7 cells

Introduction

Reactive oxygen species (ROS) produced by ultraviolet light, ionizing radiation, chemical reaction, and metabolic processes have been associated with carcinogenesis, coronary heart disease, and many other health problems related to advancing age [3,22,42]. They include superoxide radical anion ($^{\bullet}O_2^-$), hydroxyl radicals ($^{\bullet}OH$), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2). The damaging action of the hydroxyl radical is the strongest among free radicals [21]. Antioxidants can scavenge reactive oxygen species, which might have the potential to damage cell componets, such as DNA, proteins, and lipids. Oxidative damage might be involved in initiative events in cancer, and free radicals may help to induce the inhibition of apoptosis [7,8,14,19].

Cancer chemoprevention is defined as the use of chemicals or dietary components to block, inhibit, or reverse the development of cancer in normal or preneoplastic tissue [46]. One mechanism responsible for the protective role of fruit and vegetable consumption is the induction of phase 2 xenobiotic metabolizing enzymes, which include quinone reductase (QR), glutathione *S*-transferase (GST), and UDP-glucuronosyltransferase (UGT) [37]. Phase 2 enzyme induction has emerged as an important strategy for cancer chemoprevention [35]. Based on their known pattern of enzyme induction, compounds that induce drug-metabolizing enzymes are classified into bifunctional inducers that elevate both phase 1 and 2 enzymes and monofunctional inducers that selectively elevate phase 2 enzymes. Monofunctional inducers of phase 2 enzymes are considered to have a greater potential as anticarcinogenic agents because they do not enhance the activation of carcinogens mediated by phase 1 enzymes such as cytochrome P-450 [31].

Papaya (*Carica papaya* L.) belongs to the family of Caricaceae grown in Australia, Hawaii, Philippines, SriLanka, South Africa, India, Bangladesh, Malaysia and a number of other countries in tropical America [28]. Papaya sarcocarps, seeds and peels have been used traditionally to treat various ailments in humans across the world. Papaya seed is found to be a rich source of biologically active isothiocyanate [25]. Particularly, the seeds are used as emmenagogue, thirst quenchers, carminatives or for bites and stings

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of poisonous insects [50]. Benzyl isothiocyanate (BITC), a chemopreventive phytochemical found in cruciferous vegetables [49] has also been shown to be present in different extracts of papaya seeds [17,38,39]. BITC exists in these plants as glucosinolate and is released by enzyme myrosinase-induced hydrolysis when the cellular/tissue integrity of the plants is disrupted by chopping during food preparation, seed crushing and other mechanical influences [41].

In this study, antioxidant activity of methanol extracts from papaya seed and its fractions were investigated *in vitro*. As an approach to the identification of new natural cancer chemopreventive agents, methanol extracts from papaya seed and its fractions were tested for their ability to induce QR in cultured Hepa1c1c7 murine hepatoma cells and Ah-receptor-defective mutant of Hepa1c1c7 (BP^rc1 cells).

Materials and Methods

Chemicals

All chemicals were obtained from Sigma Chemical (St. Louis, Mo., U.S.A) unless otherwise indicated. Cell culture reagents were purchased from Gibco BRL (Rockville, Md., U.S.A.) and fetal bovine serum (FBS) was from Hyclone (Logan, Utah, U.S.A.). Hepa1c1c7 cells were from the American Type Culture Collection (Rockville, Md., U.S.A.), and BP^rc1 cells were kindly provided by Dr. Jong-Sang Kim (Dept. of Animal Science and Biotechnology, Kyungpook Natl. Univ., Daegu, Republic of Korea).

Preparation of samples

Papaya sarcocarp, seed and peel were extracted with methanol, and the methanol extract of papaya seed was then partitioned with n-hexane, chloroform, ethyl acetate, n-butanol, and water. The extract and solvent fractions were concentrated under reduced pressure and lyophilized. The yields (w/w) obtained for the methanol extract and its n-hexane, chloroform, ethylacetatd, n-butanol and water fractions were about 1.6%, 2.8%, 1.4%, 0.5%, 8.0% and 61.1%, respectively.

Determination of total polyphenols and total flavonoids

The concentrations of total polyphenols and total flavonoids were measured by the Folin-Denis method [11] and the method described by Nivea Moreno and other [27], respectively. Total polyphenols and total flavonoids contents were expressed as tannic acid and quercetin molar equivalents, respectively.

Scavenging of α-α-diphenyl-β-picrylhydrazyl (DPPH) radical

The free radical scavenging activity of sample was measured by the α-α-diphenyl-β-picrylhydrazyl (DPPH). This assay was carried out as described by Blois [2] with some modifications. In its radical form, $DPPH^{\bullet}$ absorbs at 517 nm but upon reduction by antioxidant or a radical species its absorption decreases. Briefly, 0.15 mM solution of $DPPH^{\bullet}$ in ethanol was prepared and 200 μl of this solution was added to 800 μl of sample solution in ethanol at different concentrations. After 30 min, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity [13]. The free radical scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$DPPH^{\bullet}$ scavenging effect (%)=$(A_{Control}-A_{Sample}/A_{Control})\times100$

Scavenging of ABTS radical

The spectrophotometric analysis of $ABTS^{+\bullet}$ radical scavenging activity was determined according to the method of Re et al. [33]. $ABTS^{+\bullet}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 24 hr. Before usage, the $ABTS^{+\bullet}$ solution was diluted to get an absorbance of 0.70±0.02 at 732 nm with phosphate buffer (0.1 M, pH 7.4). Then, 990 μl of $ABTS^{+\bullet}$ solution was added 10 μl of sample. After 1 min, the percentage inhibition at 732 nm was calculated for each concentration relative to a blank absorbance.

Scavenging of hydrogen peroxide radical

The scavenging of hydrogen peroxide radical were measured by method described by Muller others [24]. Sample 20 μl and 1 mM H_2O_2 in phosphate buffered saline (PBS) 100 μl incubated for 5 min at room temperature. And then 1.25 mM ABTS 30 μl and 1 unit/ml peroxdase 30 μl were added. After 10 min at 37℃, amount of H_2O_2 was determined by measuring absorption at 532 nm.

Determination of QR activity in cell culture

We first determined the dose-dependent characterisitics of the cytotoxic effects of the extract by means of a 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hepa1c1c7 and BP^rc1 cells were cultured as described previously [31]. In brief, cells were grown in

96-well plates at a density of 1×10^4/ml in 200 μl of α-MEM containing 100 unit/ml of penicillin G sodium salt, 100 μg/ml of streptomycin sulfate, and 250 ng/ml of amphotericin B supplemented with 10% FBS at 37℃ in a 5% CO_2 atmosphere. After a preincubation period of 24 hr, the medium was changed, lyophilized samples dissolved in DMSO were added, and the plates were incubated for an additional 48 hr. QR activity was determined by measuring the NAD(P)H-dependent menadiol-mediated reduction of MTT to blue formazan. Cellular protein was determined using a bicinchonic acid (BCA) protein assay kit (Sigma Chemical) in an identical set of test plates. The induction of QR activity was calculated from the ratio of specific enzyme activities of sample-treated cells in comparison with a nontreated control.

Results and discussion

Determination of total polyphenols and flavonoids

The content of total polyphenols and flavonoids contained in the methanol extracts from sarcocarp, seed and peel of papaya were measured using tannic acid and quercetin respectively, as standard compounds (Table 1). Contents of total polyphenols in methanol extract of sarcocarp, seed and peel were 4.67, 25.47, 7.99 μg/mg and total flavonoids contents were 1.10, 3.92, 2.62 μg/mg, respectively. The methanol extract of papaya seed contained higher levels of total polyphenols and flavonoids than papaya sarcocarp and peel. Therefore, we investigated antioxidant capacity and quinone reductase activity using methanol extracts of papaya seed and its fractions. Total polyphenols and flavonoids contents in fractions of papaya seed varied from 17.74 to 125.99 μg/mg and from 1.6 to 32.69 μg/mg, respectively (Table 2). Phenolic compounds are secondary metabolites which are widely distributed in the plant kingdom, they have diverse structures and molecular weights, and their major composition is flavonoids and tannin. The contents of total phenolics were relatively high and comparable to other vegetables and medicinal plants, which have shown pharmacological potential such as antioxidant and chemopreventive effect [16,20]. Since they contain phenolic hydroxyl (OH) groups, they bind readily to proteins and macromolecules, and they have antioxidation, anticancer, and various other physiological activities [34]. Flavonoids and other polyphenols can modulate phase II metabolism, in part via an impact on signal transduction pathways that affect the antioxidant-response element [12,40].

Table 2. Contents of total polyphenols and flavonoids in methanol extracts and fractions of papaya seed

Sample	Total polyphenols[1)] (μg/mg)	Total flavonoids[2)] (μg/mg)
MeOH extract	25.47±7.24[3)]	3.92±1.23
Hexane fraction	17.74±4.33	3.17±0.71
$CHCl_3$ fraction	31.9±3.51	5.18±0.82
EtOAc fraction	125.99±12.97	32.69±0.58
BuOH fraction	69.08±13.47	4.93±0.68
Water fraction	19.15±4.46	1.6±0.18

[1)]Micrograms of total polyphenol content/mg of plants based on tannic acid as standard.
[2)]Micrograms of total flavonoid content/mg of plants based on quercetin as standard.
[3)]Each value is mean±SD (n≥3).

Antioxidant capacity of methanol extracts of papaya seed and its fractions

We examined the antioxidant activities of the methanol extract and its fractions of papaya seed by measuring their abilities to transfer hydrogen to the stable free radicals DPPH, ABTS and H_2O_2. In these experiments, BHA, ascorbic acid and trolox were used as control compounds, respectively. The radical-scavenging activities, as indicted by their RC_{50} values, differed between the methanol extract and the various solvent fractions (Table 3), and the scavenging activity increased in a concentration-dependent manner for all radical species (data not shown). The results indicate that

Table 1. Contents of total polyphenols and flavonoids in methanol extracts from sarcocarp, seed and peel of papaya

Plant	Part used	Total polyphenols[1)] (μg/mg)	Total flavonoids[2)] (μg/mg)
Papaya	sarcocarp	4.67±0.14[3)]	1.10±0.25
	seed	25.47±7.24	3.92±1.23
	peel	7.99±0.55	2.62±0.66

[1)]Micrograms of total polyphenol content/mg of plants based on tannic acid as standard.
[2)]Micrograms of total flavonoid content/mg of plants based on quercetin as standard.
[3)]Each value is mean±SD (n≥3).

Table 3. Scavenging effects of methanol extracts and fractions of papaya seed on α, α-diphenly-β-picrylhydrazyl radicals (DPPH·), ABTS radicals and hydrogen peroxide (H_2O_2)

Sample	Fraction	RC_{50}[1] (μg/ml)		
		DPPH	ABTS	H_2O_2
Papaya seed	MeOH extract	66.28±3.65	116.52±4.86	121.69±9.33
	Hexane fraction	82.44±2.04	275.71±6.38	241.29±12.97
	$CHCl_3$ fraction	55.34±5.53	109.29±3.58	193.65±9.82
	EtOAc fraction	10.95±1.02	19.75±0.47	41.17±2.22
	BuOH fraction	21.82±1.47	53.94±7.60	49.13±4.65
	Water fraction	103.84±2.34	249.67±11.23	167.86±8.69
	BHA	3.11±1.15	-	-
	Ascorbic acid	2.09±1.03	-	-
	Trolox	-	24.57±2.35 μM	33.96±3.03 μM

[1]Concentration required for 50% reduction of free radicals at 30 min after starting the reaction.
[2]Each value is mean±SD (n≥3).

among the fractions of papaya seed, the EtOAc fraction was the most potent scavenger of DPPH free radicals (RC_{50}=10.95±1.02 μg/ml). Furthermore, the EtOAc fracion was the strongest antioxidant amongst the various fractions of papaya seed in the ABTS and H_2O_2 assay system, with RC_{50} of 19.75±0.47 μg/ml and 41.17±2.22 μg/ml, respectively. The EtOAc fraction exhibited lower RC_{50} values than trolox, indicating better radical-scavenging activity, whereas the methanol extract and the hexane, chloroform and water fractions had higher RC_{50} values and, thus, relatively low activity.

According to our results, the methanol extract of papaya seed contained higher levels of total polyphenols and flavonoids than papaya sarcocarp and peel. Contents of polyphenol and flavonoid in ethyl acetate fraction of papaya seed extract was found to be extremely high compared with the other fractions examined. In addition, the fraction showed the highest radical-scavenging activity in various antioxidant system. The result of DPPH scavenging activity assay in this study indicates that the EtOAc fraction was potently active. This suggests that the fraction contain compounds that are capable of donating hydrogen to a free radical in order to remove odd electron which is responsible for radical's reactivity. The ability of this plant extract to scavenge DPPH could also reflect its ability to inhibit the formation of ABTS+. The scavenging activity of ABTS+ radical by the plant extract was found to be appreciable; this implies that the plant extract may be useful for treating radical related pathological damage [45]. H_2O_2 is formed *in vivo* by a variety of enzymes, including superoxide dismutase. H_2O_2 is most generally considered as a powerful oxidizing agent. There is increasing evidence that H_2O_2, either directly or indirectly via its reduction product OH^-, acts as a messenger molecule in the synthesis and activation of inflammatory mediators [1]. Hydrogen peroxide itself is not very reactive; however, it can sometimes be toxic to cells because it may give rise to hydroxyl radical within the cells. H_2O_2 to cells in culture can lead to transition metal ion-dependent OH radicals mediating oxidative DNA damage. Thus, removing hydrogen peroxide is very important for protection of pharmaceuticals and food systems [4].

Induction of quinone reductase in Hepa1c1c7 cells and BPrc1 cells by methanol extracts of papaya seed and its fractions

QR is primarily a cytosolic flavoprotein that catalyzes the reduction of a wide variety of quinones and quinoneimines. QR protects cells against the toxicity of xenobiotics by promoting the obligatory 2-electron reduction of quinones to hydroquinones, which are then susceptible to glucuronidation and excretion [23]. Therefore, an assay for QR induction *in vitro* represents a simple method for screening compounds for potential anticarcinogenic properties. Assessing QR activity as a biomarker of phase II metabolism has commonly been employed to screen the potential chemopreventive activity of phytochemicals [32]. Most flavonoids show antioxidative activity and, therefore, are likely to prevent carcinogenesis by inducing detoxifying enzymes such as quinone reductase and glutathione reductase [30,43,51]. The induction of QR in cultured Hepa1c1c7 cells has been used to assess the potential of compounds from edible sources such as sulforaphane from broccoli [52] and resveratrol from grapes [15]. Also, one of the most intensively studied ITCs with regard to cancer chemo-

Table 4. Induction of quinone reductase (QR) and cytotoxic effects mediated by a methanol extract of papaya seed and its fractions in Hepa1c1c7 cells

Sample	CD[a] (mg/ml)	IC_{50}[b] (mg/ml)	CI[c]
MeOH extract	0.160	0.226	1.413
Hexane fraction	0.027	0.224	8.296
$CHCl_3$ fraction	0.086	0.133	1.547
EtOAc fraction	0.028	0.180	6.429
BuOH fraction	0.031	0.104	3.355
Water fraction	0.079	0.463	5.861

[a]Mean value of the concentration required to double the specific activity of QR.
[b]Mean value of the half-maximal inhibitory concentration of cell viability.
[c]The ratio between IC_{50} and CD.

prevention is benzyl isothiocyanate (BITC), a product of enzymatic hydrolysis of glucotropaeolin. BITC is contained in high amounts in papaya (*Carica papaya*), garden cress (*Lepidium sativum*) and common Brassica vegetables [10,29,39,44] and some studies show the potential of this compound as a chemopreventive agent in man [5,6,47-49,53].

Both epidemiological studies and *in vitro* and *in vivo* experiments have found that various compounds from many plant sources, mostly fruits and vegetables, offer protection against various types of cancer. Among many vegetables, cruciferous vegetable belong to the species Brassica (for example, broccoli, cabbage, cauliflower, kale) have been known as important dietary contributors to cancer prevention. In general, broccoli and brussels sprouts have been known as the most potent sources. Cruciferous vegetables are the best dietary source of health promoting glucosinolate conversion products such as sulforaphane [26,35] and their protective effect against cancer has been attributed to the induction of phase 2 enzyme such as QR [9].

To obtain further information relative to the characteristics of the active compound(s) responsible for QR induction potency, the methanol extract was fractionated using a series of solvents of increasing polarity. CDs (the concentration required to double the specific activity of QR), IC_{50}s and CIs (the ratio between IC_{50} and CD) obtained for methanol extracts of papaya seed and various solvent fractions are shown in Table 4. The CDs obtained for the hexane and EtOAc fractions were about 1.2 to 5.9-fold lower than those obtained for the other fractions, and CDs for all of the fractions were lower than that obtained for the methanol extract, implying that these fractions had higher induction activities for QR than the methanol extract. The IC_{50}s, an indication of cytotoxicity, obtained for the fractions, except the water fraction, were about 0.1-0.2 mg/ml, whereas the IC_{50} obtained for the water fraction was much higher than that of the others. Although the CI obtained for the hexane fraction was higher than those obtained for the others, this result was due to its lower cytotoxicity in Hepa1c1c7 cells. The EtOAc fraction from papaya seed induced QR activity in the concentration 12.5 to 50 μg/ml with a maximum of a 3.3-fold induction at 50 μg/ml of fraction (Fig. 1). But the hexane fraction induced QR activity with a maximal 2.0-fold increase at a concentration of 25 μg/ml.

To determine whether the extract is a monofunctional inducer of QR, its ability to increase QR activity in the mutant cell line of Hepa1c1c7, BP^rc1, was examined. As shown in Figure 2, the methanol extract of papaya seed and its fractions did not induce QR activity in the mutant BP^rc1 cells, and this indicates that the mutant BPrc1 cell line used in this experiment responded in the predicted manner to a

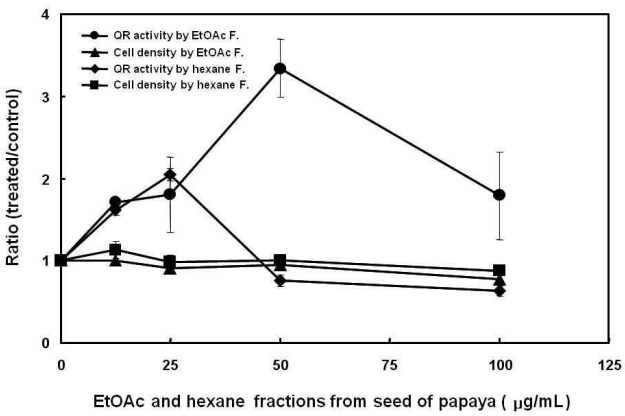

Fig. 1. Induction of quinone reductase (QR) in Hepa1c1c7 cells by EtOAc and hexane fractions of papaya seed. Cells were treated with the fraction in a concentration range of 0 to 100 μg/ml for 48 hr. Each value represents the mean±standard deviation of 3 independent experiments and is expressed relative to a vehicle control.

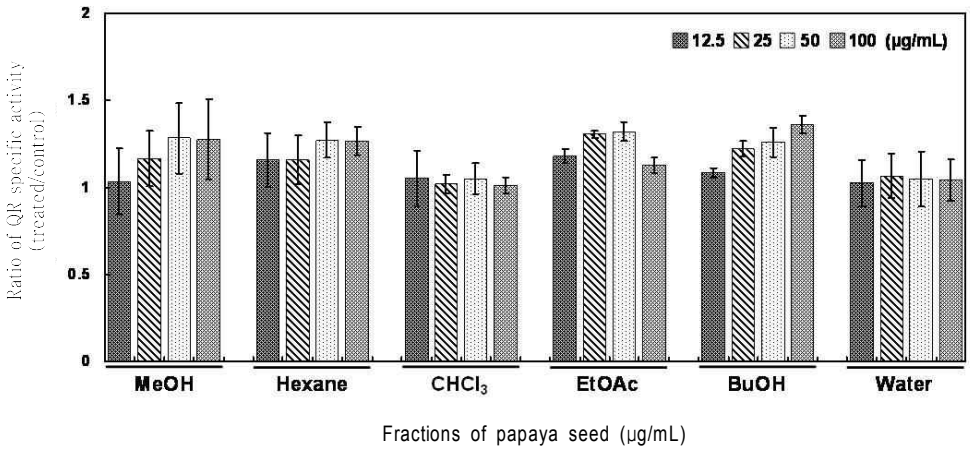

Fig 2. Quinone reductase (QR) activity of Ah-receptor-defective mutant of Hepa1c1c7 cells (BPrc1) treated with methanol extracts and fractions of papaya seed. Cells were treated with the fraction in a concentration range of 12.5 to 100 μg/ml for 48 hr. Each value represents the mean±standard deviation of 3 independent experiments and is expressed relative to a vehicle control.

well-characterized bifunctional inducer. Mutant cell line of Hepa1c1c7, BP^{r}c1 cells lacking arylhydrocarbon receptor nuclear translocator (ARNT), which are typical murine hepatoma cell lines highly responsive to phase 2 enzyme inducers and thereby widely used for screening phase 2 enzyme inducers [18]. The methanol extract of papaya seed and its fractions did not induce QR activity in the mutant BP^{r}c1 cells (Fig. 2).

Overall, the data indicate that papaya seed has a positive impact on the phase II enzymes QR, suggesting that they may enhance detoxification of carcinogens/toxins and their removal from the body. Various compounds, natural and synthetic, are capable of elevating phase II enzyme activity and are classified as monofunctional (phase II) inducers [31]. Bifunctional inducers have the capacity to increase both phase I and phase II enzymes. These data are consistent with the finding of Talalay *et al.* [36] that the flavonoids, which occur abundantly in many common edible plants, are typical bifunctional inducers.

Therefore, the most effective QR inducer among these fractions is resided in the EtOAc fraction, indicating that strong constituents responsible for QR induction potency in the papaya seed extract are largely contained in the EtOAc fraction.

Acknowledgements

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST) (No. 353-2009-1-F00018), and the Grant of the Korean Ministry of Education, Science and Technology (The Regional Core Research Program/Anti-aging and Well-being Research Center).

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초록 : 파파야씨 추출물 및 분획물의 항산화, QR 활성

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본 연구에서는 파파야씨의 메탄올 추출물과 분획물을 이용하여 항산화 활성 및 quinone reductase (QR) 활성을 측정하였다. 파파야씨의 추출물 및 분획물의 총 폴리페놀 함량은 17.74~125.99 μg/mg이며, 총 플라보노이드 함량은 1.60~32.69 μg/mg으로 나타났으며, ethyl acetate (EtOAc) 분획층의 총 폴리페놀 및 플라보노이드 함량이 다른 분획층과 비교했을 때 가장 높은 것으로 나타났다. α,α-Diphenyl-β-picrylhydrazyl (DPPH) radicals, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) and hydrogen peroxide를 이용하여 파파야씨 메탄올 추출물 및 분획물의 항산화 활성을 조사한 결과, EtOAc 분획층에서 가장 높은 free radical 억제능을 보였다. 또한 EtOAc 분획층 12.5~50 μg/ml의 농도에서 QR의 유도활성을 조사한 결과, 50 μg/ml의 농도에서 3.3배 정도의 QR 유도능을 보였다. 따라서 파파야씨의 EtOAc 분획층에 존재하는 물질들은 QR inducer 로써의 역할이 기대된다.