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Antioxidative and Antiobesity Activity of Nepalese Wild Herbs†

Amrit Poudel, Se-Gun Kim, Do Kuk Kim, Yun Kyung Kim, Young Suk Lee[1], Gye Won Lee[2], Byung-Sun Min[3] and Hyun-Ju Jung*

Department of Oriental Pharmacy and Center for Animal Resource Development, Wonkwang University, Sinyong-dong, Iksan 570-749, Korea
[1]Department of Pharmacy, Wonkwang University, Sinyong-dong, Iksan 570-749, Korea
[2]Laboratory of Galenic Technology, Department of Pharmaceutical Engineering, Konyang University, Daehak-ro 119, Nonsan 320-711, Korea
[3]College of Pharmacy, Catholic University of Daegu, Gyeongbuk 712-702, Korea

Abstract – A screening of Nepalese wild herbs for their antioxidant and antiobesity activity was carried out. The herbs including *Allium hyposistum, Crateva unilocularis, Dryoathyrium boryanum* and *Cuscuta reflexa* are widely used traditionally for various medicinal purposes in Nepal. The ethyl acetate fraction of *D. boryanum* showed polyphenol content of 266 μgGAE/mg with potent antioxidative activity assessed by DPPH free radical scavenging activity and hydrogen peroxide scavenging activity. The EtOAC fraction of *D. boryanum* also inhibited the lipid formation with 35% at 100 μg/mL in 3T3-L1 cell model. Along with this, butanol fraction of *C. reflexa* also showed potent antioxidative activity and inhibition of 80% of lipid formation at the test concentration of 75 μg/mL in 3T3-L1 cell line. This showed that these plant extracts have potential of antioxidant and antiobesity activity.
Keywords – Antioxidant, DPPH, Antiobesity, 3T3-L1 cell line

Introduction

Obesity is a serious health problem leading to shortened life expectancy due to the complications from resultant diseases such as heart disease, type 2 diabetes, hyperlipidemia, cancer and osteoarthritis (Park *et al.*, 2010). It is generally accepted that weight management plays a significant role in the prevention of these diseases. Therefore, much research has been conducted on the identification of natural substances that exhibit antiobestic properties. As a consequences numerous dietary supplements that promote the reduction of body weight and fat mass are now available on market (Jun *et al.*, 2010).

Reactive oxygen species or free radicals in biological systems including hydroxyl radical (·OH), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) have been associated with many age related degenerative diseases and these reactive oxygen species could be one of important links between obesity and its major associated disorders such as insulin resistance and hypertension (Kim *et al.*, 2010). Plasma lipids are oxidized at a faster rate in obese persons compared with those of non-obese persons. In obesity, elevated lipid pools in the adipose tissue depots or in the blood are targets for free radical attack (Vincent *et al.*, 2006). Moreover, the surrounding circumstances make more intensified and serious obesity as well as other obestic complications. Recent studies have suggested that dietary supplementation with radical scavenging ability may relate to reduction of body weight or several obesity related disorders.

Due to extreme geographical diversity, unique topography and climatic condition, Nepal has a wide variety of flora and fauna. Nepal is rich with approximately 5800 flowering plant species. There are about 800 plant species with reported medicinal values (Manandhar, 1998). A number of medicinal plant species are used in different traditional systems in diversified patterns from centuries ago in Nepal. Despite the reputation earned by medicinal and aromatic plants of Nepal, very little work has been reported for their chemical constituents and biological activities. So, as a continuous effort to search for new antiobesity natural product, we have tried to study and report the antioxidant and antiobesity activity of Nepalese traditional herbs from a potential virgin filed of research.

†Dedicated to professor KiHwan Bae for his leading works on bioactive natural products.
*Author for correspondence
Tel: +82-63-850-6814; E-mail: hyun104@wku.ac.kr

Allium hyposistum is an herb that is used extensively as a spice to flavor vegetable, pickles and meat in Nepal. Most of the households use this herb as a medicine for the treatment of flu. *Crateva unilocularis* is a deciduous herb. The shoot of the plant is used as vegetable and believed to help in diabetes. The fruit paste of this herb is used in case of smallpox (Manandhar, 1990). *Dryoathyrium boryanum* is an herbaceous fern. The tender shoot is cooked as vegetable and is medicinally used as demulcent, stomachic and laxative. *Cuscuta reflexa* is a holoparasite distributed throughout Nepal. The plant is believed to purify blood, clean the body and lessen inflammation. Paste of the plant is applied to relieve body pain and the juice of the plant is widely used for the treatment of jaundice (Rajbhandari, 2001). Some scientific study showed that methanolic extract of *C. reflexa* has significant anticonvulsive property by altering the level of cathecholamines and brain amino acids in mice (Gupta *et al*., 2003). The petroleum ether extract of *C. reflexa* showed reduction in muscle relaxant activity and showed analgesic properties as well (Pal *et al*., 2003). The methanol extract of *C. reflexa* stem was found to cause significant increase in clotting time in mice at moderate and high doses (Mazumder *et al*., 2003). Moreover, it showed significant antibacterial activity against *staphylococcus aureus*, *Shigella boydii*, *Pseudomonas aeruginosa*, *Shigella dysenteriae* and *Escherichia coli* (Pal *et al*., 2006).

Materials and Methods

Plant materials and Extraction – *Allium hyposistum* (aerial part), *Crateva unilocularis* (plant shoots), *Dryoathyrium boryanum* (aerial part) and *Cuscuta reflexa* (whole plant) were collected from Nepal. The plants were identified with the help of literature comparision. In addition to this, it was further confirmed by the taxonomy expert Mr. Kuber Jung Malla, Department of Plant Resources, Kathmandu, Nepal.

Hot extraction of shed dried plant parts with methanol was carried out. After filtration, the methanol was then evaporated in vacuum. So formed dry methanol extract (MeOH) was suspended in water. Fractionation with Hexane, ethyl acetate and butanol was carried out successively to give hexane extract (Hex), ethyl acetate extract (EtoAc), butanol extract (BuOH) and water soluble residue (H_2O).

Total polyphenol Content – Total polyphenol content of different fractions of plants was determined using Folin-Ciocalteu reagent according to the method of Singleton and Rossi with some modification (Singleton and Rossi, 1965). 1 N Folin-Ciocalteu reagent (1 mL) was added to 1 mg/mL plant extracts (1 mL) followed by the addition of distilled water (5 mL). After 5 min of incubation 10% Na_2Co_3 (1 mL) was added and incubated for 1 hour in the dark at room temperature. The absorbance was measured at 725 nm using UV spectrophotometer. A calibration curve of gallic acid was drawn with the concentration of 10, 50, 100, 150, 200, 250 μg/mL. Using the standard calibration curve of gallic acid the total polyphenol content of extracts were expressed as microgram of gallic acid equivalent per milligram of extracts (μg GAE/mg).

DPPH radical scavenging activity – The antioxidant capacity of different fractions of plants were confirmed by the DPPH free radical scavenging activity assay according to Hazra *et al.* with slight modification (Hazra *et al*., 2010). Different concentrations (10, 50, 100 μg/mL) of the extracts prepared in DMSO was added to 150 μM DPPH and kept in dark for 30 min. The optical density of resultant solution was measured in UV spectrophotometer at 517 nm. Ascorbic acid was used as positive control. All the measurements were repeated for three times. The percentage radical scavenging activity was measured from the following formula.

$$\text{Percentage radical scavenged} = [(A_0 - A_1) / A_0]*100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of sample.

Hydrogen Peroxide scavenging activity – This assay was based on the ability of different fractions of plant extracts to scavenge the hydrogen peroxide in ABTS-peroxidase medium according to the method of Muller (Muller, 1985). Sample (20 μl) and 1 mM hydrogen peroxide (20 μl) were mixed with phosphate buffer (100 μl) in a 96-microwell plate and incubated for 5 min. Finally freshly prepared 1.25 mM ABTS (30 μl) and 1 U/mL peroxidase (30 μl) were mixed and incubated at 37 °C for 10 minutes. The absorbance was measured in ELISA reader at 405 nm.

Cell viability assay – Cell viability assay was conducted to determine the cell toxicity effect of plant extracts on 3T3-L1 preadipocyte cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Bovine calf serum (BCS) at 37 °C in 5% CO_2. 3T3-L1 preadipocytes were placed in 48 well plates at a density of 2.5×10^4 cells/well. Cells were then cultured for 24 hours. After 24 hours, medium was changed by DMEM and plant extracts of 10, 50 and 100 μg/mL were added and cultured for another 24 hours. MTT (5 mg/mL) solution prepared in phosphate buffer saline was added to

each well and incubated for 2 hours at 37 ºC. Formazan formed by viable cells was dissolved with dimethylsulfoxide (DMSO) and absorbance was determined at 520 nm by ELISA reader. Cell viability less than control group was considered as toxic dose. If toxicity is seen, experiment was repeated in lower concentration to find non-toxic dose in 3T3-L1 preadipocyte cells.

Adipocyte differentiation of 3T3-L1 preadipocyte cells – 3T3-L1 preadipocyte cells (3 of cell passage) were cultured in DMEM with 10% BCS at 37 ºC in 5% CO_2. After post confluent, cells were exposed to differentiation medium consisting of DMEM with 10% Fetal bovine serum (FBS), 10 µg/mL insulin, 1 µM dexamethasone and 0.5 mM 3-isobutyl-1-methylxanthin for 2 days. Cells were incubated for additional 2 days in DMEM containing 10% FBS and 10 µg/mL insulin followed by culturing with 10% FBS in DMEM for an additional 4 days. On day 0, 2, and 4 plant extracts in three different non-toxic doses were treated (Yang *et al.*, 2008).

Oil red O staining – Oil red O staining was used to monitor lipid accumulation in differentiation adipocytes. Monolayer cells were washed with phosphate buffered saline (PBS), fixed with 10% formalin PBS (pH 7.4) and stained with 0.2% Oil Red O/isopropanol for 1 hour and excess of stain was removed by 60% isopropanol. Stained oil droplets were dissolved with isopropanol and quantified at 510 nm by ELISA reader (Yang *et al.*, 2008).

Results and Discussion

Total polyphenol content – Phenolic compounds constitute a group of substances that are widely present in the plant kingdom, where more than 8000 are known with different chemical structures and activities. Polyphenols have pharmacological properties such as antioxidant, antithrombotic, anti-inflammatory and anti-HIV activity (Hsu *et al.*, 2006). Several polyphenols have been shown to exert an effect on lipid catabolism, glucose transport, insulin-receptor function and peroxisome proliferator activated receptors (PPARs) activation, all of which play essential roles in obesity. It has been reported that plant polyphenols increase the activity and gene expression of enzymes involved in hepatic fatty acid oxidation in experimental animals (Fukuchi *et al.*, 2008). For the qualitative analysis the TLC plates were treated with ferric chloride solution. Then the polyphenol content was compared with gallic acid content per milligram of extract. All fractions of different extracts showed presence of certain level of polyphenol content. Ethyl acetate fraction of *C. reflexa* showed high content of 304 ± 4.45 µgGAE/mg of polyphenol. The result is as shown in Fig. 1.

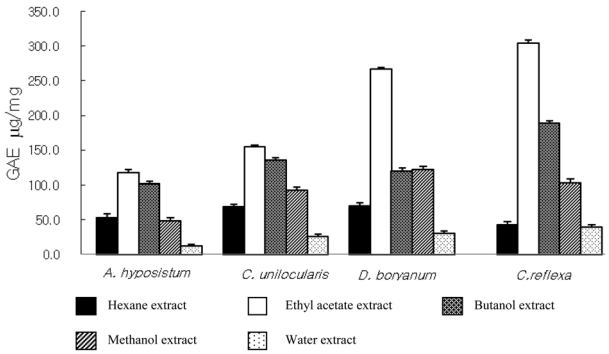

Fig. 1. Total polyphenol content of fractions of different plant extracts. Results are mean ± SD of triplicate data.

DPPH radical scavenging activity – Virtually all diseases thus far examined involve free radicals. In most cases, free radicals are secondary to the disease process, but in some instances free radicals are causal. Thus, there is a delicate balance between oxidants and antioxidants in health and disease. In normal healthy physiology, free radicals can be combated with endogenous antioxidant defenses. But in case of obesity, antioxidant defenses are compromised and several sources of free radical production might contribute to exacerbation of oxidative damage (Vincent *et al.*, 2006).

DPPH radical is a stable chemically synthesized radical, which is frequently used for the evaluation of antioxidative activity due to its convenience to handle. All the extracts exhibited certain level of DPPH radical scavenging activity and the activity increased with the increase in concentration of the extract. Ascorbic acid was taken as positive control. The result showed ethyl acetate fraction of *D. boryanum* along with ethyl acetate fraction and butanol fraction of *C. reflexa* have radical scavenging activity significantly comparable with that of ascorbic acid at the concentration of 100 µg/mL. The result is as shown in Fig. 2.

Hydrogen Peroxide scavenging activity – Hydrogen peroxide is a weak oxidizing agent that inactivates a few enzymes directly, usually by oxidation of essential thiol (-SH) groups. It can cross cell membranes rapidly; once inside the cell, it can react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radicals and this may be the origin of many of its toxic effects (Hazra *et al.*, 2010). Hydrogen peroxide in ABTS-peroxidase medium was used for the production of hydroxyl radical in the medium. Butylated hydroxyanisole (BHA) was used as positive control. Butanol fraction of *A. hyposistum* showed hydrogen peroxide scavenging activity significantly at 100 µg/mL

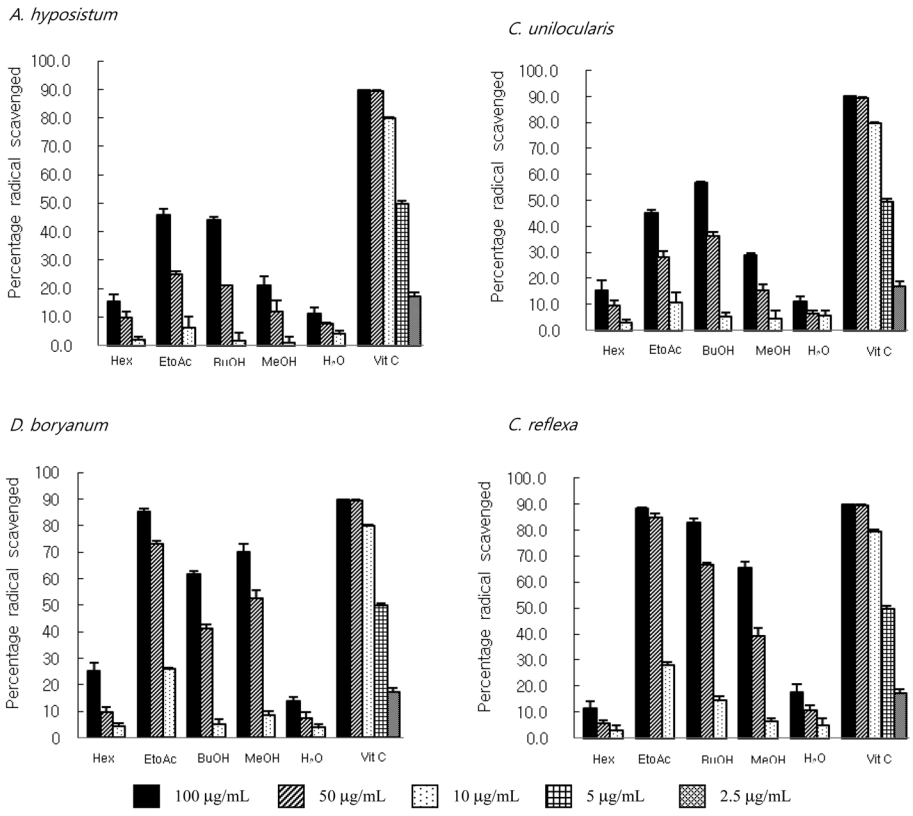

Fig. 2. DPPH radical scavenging activity of different fractions plant. Results are mean ± SD of triplicate data.

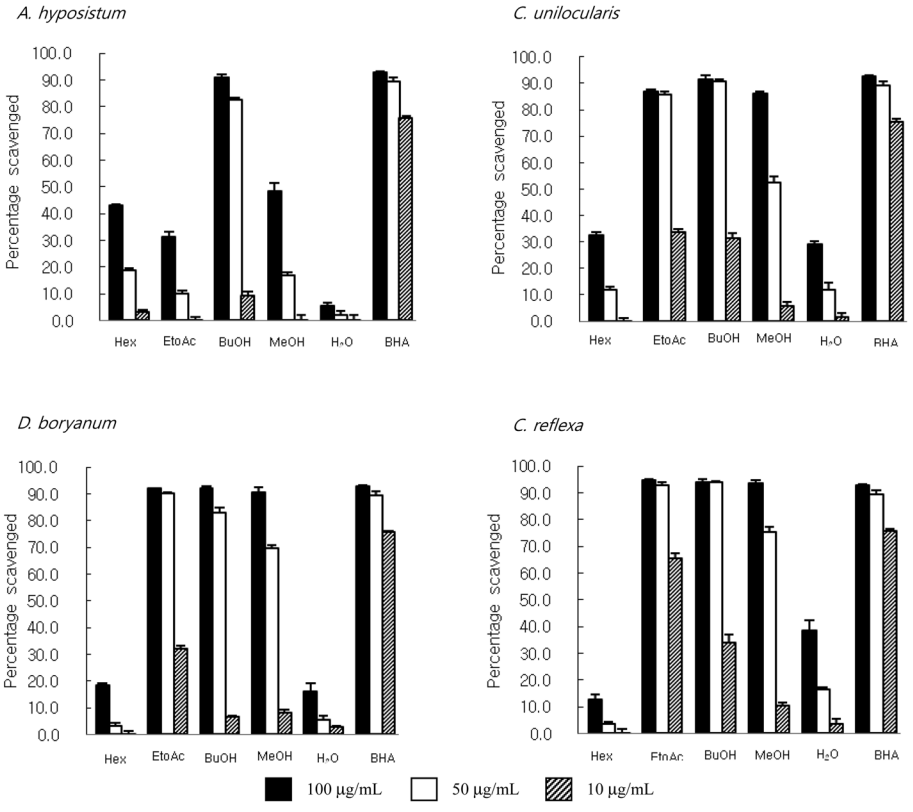

Fig. 3. Hydrogen peroxide scavenging activity of different fractions of plants. Results are mean±SD of triplicate data.

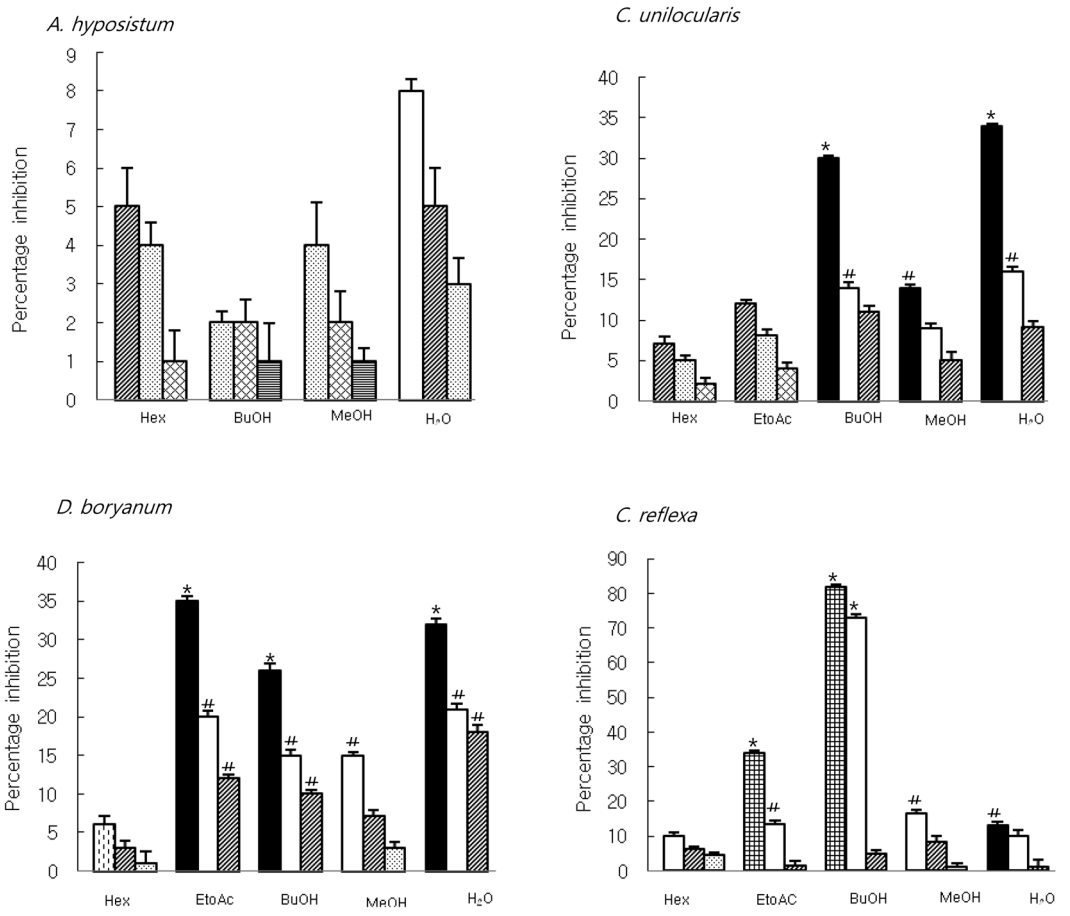

Fig. 4. Inhibition of lipid formation in 3T3-L1 cell by different fractions of plants. Results are mean±SD of triplicate data. *p < 0.005 vs control group, #p < 0.05 vs control group (studentís t-test).

comparable with that of BHA. Ethyl acetate, butanol and methanol fractions of *C. unilocularis, D. boryanum* and *C. reflexa* showed significant scavenging activity compared with BHA. Results are shown in Fig. 3.

Adipocyte differentiation of 3T3-L1 preadipocyte cells – Obesity is characterized at the cell biology level by an increase in the number and size of a adipocytes differentiated from fibroblastic preadipocytes in adipose tissues (Hsu *et al.*, 2006). 3T3-L1 cell line is a classic cell type to study adipogenesis developed through clonal expansion of rodent derived cells. It is frequently used for screening of adipogenesis potential of various agents (Poulos *et al.*, 2010). After cell viability test three different concentrations without toxicity was considered for the activity test. The ethyl acetate fraction of *A. hyposistum* was found to be very toxic to the cell so it was not considered for the test. Ethyl acetate fraction of *D. boryanum* at the concentration of 100 μg/mL showed almost 35 ± 0.57% of inhibition while butanol fraction of *C. reflexa* at the concentration of 75 μg/mL showed high inhibition of almost 80 ± 0.73% of lipid formation. The result is as shown in Fig. 4.

Various antiobesity drugs have been studied and developed such as rimonabant, metformin, exenatde, redux and fen-phen to reduce or control body weight. However, these antiobesity drugs frequently elicit serious side effects including high blood pressure, headache, constipation and insomnia. Safe and efficacious regimens based on complementary and alternative medicine could contribute significantly to weight management and reduction efforts to avert obesity (Park *et al.*, 2010). Of interest, recent studies reported an antiobesity effect of herbs with little toxicity. Obesity has been shown to be one of the conditions that decrease antioxidant capacity. Obesity seems to decrease antioxidant defense by lowering the antioxidant enzymes such as catalase, glutathione peroxidase, glutathione reductase and by altering the activity of cytochrome P-450 (Ozaydin *et al.*, 2006).

Oxidative stress is directly related with plasma malondialdehyde (MDA) level. Experiment showed an increase in plasma MDA level and decrease in vitamin-E concentration in the obese than in the non obese group (Skrha *et al*., 1999). As of the evidence, oxidative stress plays an important role in the pathogenic mechanism of obesity and obesity associated metabolic syndrome. So, in our study, the inhibition of lipid formation in cell line by ethyl acetate fraction of *D. boryanum* and butanol fraction of *C. reflexa* can be correlated with the high polyphenol content and antioxidative activity of those fractions. Chemical constituents from *D. boryanum* has not been reported yet, while different polyphenols like caffeoylquinic acid, quercetin, kaempferol has been isolated from *C. reflexa*. This experiment suggests that chemical isolation from butanol fraction of *C. reflexa* could lead to isolation of potent antioxidant and antiobesity active compounds.

Conclusion

Antioxidative activity by DPPH free radical scavenging activity and hydrogren peroxide scavenging activity along with antiobesity activity by inhibition of lipid accumulation in 3T3-L1 cell model of hexane, ethyl acetate, butanol, methanol and water fractions of *A. hyposistum, C. unilocularis, D. boryanum* and *C. reflexa* was carried out. Ethyl acetate fraction of *D. boryanum* and *C. reflexa* showed significantly high content of polyphenol. Ethyl acetate fraction of *D. boryanum* showed potent antioxidative activity in dose dependent manner and it showed an inhibition of lipid formation in 3T3-L1 cell model by 35% while Ethyl acetate and butanol fraction of *C. reflexa* showed potent antioxidative activity in dose dependent manner and the butanol fraction showed an inhibition of lipid formation by 80% in 3T3-L1 cell model. The antiobesity activity of these plants in 3T3-L1 cell model was reported for the first time.

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