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# Estimation of Antioxidants (Flavonoids) in *Ailanthus excelsa and Balanites aegyptiaca* and Effect of Growth Regulators and Salts on Antioxidants *in vitro*

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**Abstract:** ***Ailanthus excelsa*** **and** ***Balanites aegyptiaca*** **are two wild growing common plant species of desert, having medicinal importance. Unorganized tissues of these useful plants were established on MS medium supplemented with 1.0mg/L BAP+1.5mg/L 2,4-D and 1.5mg/L BAP +2.0mg/L 2,4-D respectively (standardized MS medium). Parts of established tissues were transferred to standardized (Sd) MS medium fed with various concentrations(1,2,3 mg/L) of growth regulators ( IAA, NAA) and (10,20,30mg/L) salts ( NaCl , KCl ) separately. Tissues at the maximum GI( in all samples) were harvested, dried, powdered and analyzed for estimation of flavonoids. Maximum amount of flavonoids was calculated in callus fed with 1mg/L IAA, NAA and 10 mg/L NaCl and KCl in *A. excelsa* and *B. aegyptiaca*.**

**Keywords:** *Ailanthus excelsa , Balanites aegyptiaca,* antioxidants, growth regulators, salts.

## 1. Introduction

**Ardu *(Ailanthus excelsa)*** belonging to family **Simaraubaceae** is a large deciduous tree having a lot of medicinal uses. It is a native of India and Sri Lanka. The plant is recognized for its varieties of medicinal uses like anticancer, contraceptive, post partum treatment, intestine tape worm, dysentery, epilepsy, heart troubles, asthma etc. The plant also having antibacterial, antimicrobial and antifungal activity. Two new tetracyclic triterpenes (ailexcelone and ailexcelol) were isolated from the heartwood of ***Ailanthus excelsa*** which are tested for their antifungal activity ( Srinivas *et al,* 2006).

***Balanites eagyptiaca*** **" Desert Date"** of family **Zygophyllaceae** is a common wild plant found in many kind of habitates ,tolerating a wild variety of soil types from sand to heavy clay and climatic moisture levels. It is believed indigenous to all dry lands.

The root, stem bark ,fruit pulp and kernel cake of ***Balanites eagyptiaca*** have medicinal properties. Fruit is used in whooping cough also in leucoderma and other skin diseases. It is used as an oral hypoglycemic and an antidiabitic .An aqueous extract of the fruit mesocarp is used in Sudanese folk medicine in treatment of jaundice. It is also used to treat dysentery and constipation. Fruit is used to treat liver diseases and as a purgative and sucked by school children as a confectionary in some countries.

***Balanites eagyptiaca*** contains steroids ( saponins, sapogenins, diosgenins ) used as raw material for industrial production of contraceptive pills, corticoids, anabolisants and other sexual hormones. The saponins occurring in roots ,woodchips and fruits facilitate their use for washing clothes..

Medicinal plants are rich source of secondary metabolites, but restricted to specific taxonomic genera of plant kingdom and specific part of plant body. Secondary metabolites present in small quantities in specialized cells, but they possess significant biological activities ranging from antibacterial, antibiotic, insecticidal, hormonal, pharmacological and pharmaceutical.

**Antioxidant** is simply a molecule that prevents another molecule from oxidizing. Since there are many processes in the body which result in oxidation . The intake of antioxidant is essential to counteract some of the negative results of the buildupof too many oxidized molecules in the body.

**Flavonoids** are a type of antioxidants ,water soluble phenolic glycosides, which occur almost universally in higher plants. They are generally not synthesized by the animals. Flavonoids are easily recognizable as the pigments in flowers and fruits. They have multiple biological effects including antioxidant free radical scavenging abilities, anti-inflammatory , anticarcinogenic antiallergic, antiulcer, antihepatotoxic, antiviral, antianginal, antispasmolytic etc. Their contribution to physiological functions such as seed maturation and dormancy has already been established ( Brenda, 1998 ).Their vital role is defences against pathogens and predators and physiological functions ( Winkel Shirley, 2001,2002 ).

Presence of flavonoids *in vitro* has been reported from many plant species like ***Embilica officinalis*** (Kamal *et al*.,1982 ),***Stevia neplifolia*** (Rajbhandari ,1984), ***Arachis hypogea*** (

Pratt,1984), ***Tribulus alatus*** ( Jit and Nag, 1985), ***Peganum harmala*** ( Badia,1999) , ***Vigna aconitifolia*** (Tyagi, 2002), ***Calligonum*** and ***Withania somnifera*** (Bains, 2002), ***Capparis decidua*** and ***Zizyphus mauritiana*** (Chauhan, 2003), ***Cassia angustifolia*** (Goswami and Reddy, 2005), ***Balanites aegyptiaca*** (Bedawat, 2006), ***Ailanthus excelsa*** (Rao, 2007), medicinal plants ( Goswami *et al.*),***Pueraria tuberosa*** (Goyal and Ramawat, 2008), ***Azadirachta indica*** (Babu *et al.,* 2008) ***Adhatoda vasica*** and ***Barleria prionitis*** (Deepa, 2009), ***Cocculus pendulus*** and ***Tinospora cordifolia*** (Yadav, 2010), ***Aegle marmelos*** (Sharma,2010), ***Terminalia arjuna*** ( Sharma, 2012), medicinal plants (Talreja *et al*.,2012), ***Maytenus emarginata*** ( Mathur, 2013) ***Moringa oleifera*** (Petchang, 2014 ), ***Aegle marmelos*** and ***Moringa oleifera*** ( Soni,2015).

## 2. Materials and Methods

Unorganized tissue of ***Ailanthus excelsa*** and ***Balanites aegyptiaca*** were established on MS medium supplemented with 1mg/L BAP+1.5mg/L 2,4-D and 1.5mg/L BAP +2mg/L 2,4-D respectively ( standardized MS media for both species). Parts of established tissues were transferred to standardized (Sd) MS medium fed with various concentrations (1,2,3 mg/L) of growth hormones ((IAA, NAA) and (10, 20, 30mg/L) salts ( NaCl , KCl ) separately. GI was calculated in all samples.Tissues at the maximum GI(grown on Sd MS medium, Sd MS medium with all variations of growth regulators and salts in both plant species ) were harvested, dried, powdered and analyzed for estimation of flavonoids.

### 2.1 Extraction Procedure

The dried samples were separately soxhlet extracted by **Subramanian and Nagarajan (1969)** method, in 80% ethanol (100 ml/g.d.w.) on a water bath for twenty four hours. Each of the extract was concentrated and re-extracted in petroleum ether (40-60 C, fraction first), ethyl ether (fraction second) and ethyl acetate (fraction third) in succession. Each step was repeated three times to ensure complete extraction in each case. Fraction first was rejected due to its richness in fatty substances, whereas fraction second was analyzed for free flavonoids and fraction third for bound flavonoids in each of the samples.

Fraction third of each of the test samples was hydrolyzed by refluxing with 7% sulphuric acid (10 ml/gm residue) for two hours. The mixture was filtered and the filtrate was extracted with ethyl acetate in separating funnel. The ethyl acetate layer (upper layer) was washed with distilled water to neutrality, dried *in- vacuo* and analyzed for bound flavonoids.

### 2.2 Qualitative Analysis

Thin glass plates coated with silica gel G were dried, activated at 100 oC for 30 minutes and cooled at room temperature. Ethyl ether and ethyl acetate fractions from each of the test sample were separately applied 1 cm above the edge of the plates along with the standard reference compounds (apigenin, isorhamnetin, scutellarein, kaempferol, luteolin, quercetin, myricetin, scopoletin and negretin).These glass plates were developed in solvent mixture of n-butanol, acetic acid and water (4:1:5,upper layer).

The developed plates were air dried and visualized under UV light (254 nm) which showed two and one fluorescent spots ( in ***A. excelsa*** and ***B. eagyptiaca*** respectively ) in ethyl ether fraction (second) coinciding with those of the standard samples of quercetin (yellow, $R_f$ 0.82), luteolin ( yellow, $R_f$ 0.78) and one spot in ethyl acetate fraction (third) in both ***A. excelsa*** and ***B. eagyptiaca*** coinciding with those of the standard samples of kaempferol (deep yellow, $R_f$ 0.93). The plates were also placed in a chamber saturated with ammonia vapors to observe the colors of the spots (quercetin–yellow, luteolin-yellow and kaempferol–deep yellow). On spraying the developed plates with 5% ethanolic ferric chloride solution two spots (quercetin,luteolin) and one fluorescent spot ( quercetin) in ***A. excelsa*** and ***B. eagyptiaca*** respectively were seen in ethyl ether fraction (second) and one spot in ethyl acetate fraction (kaempferol) in both ***A. excelsa*** and ***B. eagyptiaca.*** The Rf values were calculated as an average of the five replicates.

### 2.3 Preparative Thin Layer Chromatography (PTLC)

Glass plates (20 x 20 cm), thickly coated (wet thickness, 0.4-0.5 mm) with silica gel 'G' (45 gm / 80 ml water), activated at $100^0$C for 30 minutes and cooled at room temperature, were used for preparative thin layer chromatography (PTLC). The extract of both the fractions (second and third) of ***A. excelsa*** and ***B. eagyptiaca*** were applied on separate plates and developed plates were air dried and visualized under UV light (254 nm). Each of the fluorescent spot coinciding with those of the standard reference compounds of quercetin, luteolin and kaempferol were marked. The marked spots were scrapped and collected separately along with the silica gel and eluted with ethanol. Each elute was then crystallized with chloroform.

The compounds thus isolated were subjected to colorimetry (for quantitative estimation), melting point (melting point apparatus, Toshniwal, India), UV maxima on a spectrophotometer (Carl-zeiss, Jena, DDR, VSU-2P) and Infra-red spectral (Perkin-Elmer, 337, Grating Infra-red spectrophotometer, using nujol or potassium bromide pellets) studies.

### 2.4 Quantitative Analysis

**Spectrophotometry**

Quantitative estimation of the identified flavonoids was carried out colorimetrically following the method of **Kariyon** *et al.,* **(1953)** and **Naghski** *et al.,* **(1975)** in case of quercetin as well as luteolin and of **Mabry** *et al.,* **(1970)** in case of kaempferol. Stock solutions of quercetin, luteolin and

kaempferol were separately prepared by dissolving the authentic samples in methanol. Six concentrations (25 mg/ml to 150 mg/ml) of each of the standard samples were spotted on silica gel 'G' coated and activated plates. Separate plates for each of the concentrations of quercetin, luteolin and kaempferol were used and these chromatograms were developed in the same solvent system as used for qualitative method (n-butanol:acetic acid: water, 4:1:5 ; upper layer). Such developed chromatograms were air dried and visualized under UV light (254 nm). The fluorescent spots were marked and collected along with the absorbent in separate test tubes in methanol. The mixture was shaken vigorously, centrifuged and supernatants collected separately. The volume of elutes was made up to 10 ml by adding spectroscopic methanol. To each of these samples 3 ml of 0.1 M aluminium chloride was added stoppered tightly and the mixture shaken vigorously. Such tubes were kept at room temperature for 20 minutes. Five such replicates were prepared in each case and optical densites (O.D.) measured, using spectronic 20 colorimeter (Bausch and Lomb), set at 440 nm for quercetin, luteolin and at 423 nm for kaempferol against a blank (10 ml spectroscopic methanol + 3 ml of 0.1 M $AlCl_3$). Regression curves for quercetin, luteolin and kaempferol were separately plotted in between their respective concentrations and optical densities, which followed **Beer's** law.

Each of the ethyl ether and ethyl acetate extracts was dissolved in 1 ml of spectroscopic methanol and applied (0.1 ml) on silica gel 'G' coated plates along with authentic quercetin, luteolin and kaempferol markers and developed as above. Fluorescent spots coinciding with those of the reference compounds were marked, scrapped, eluted with methanol separately and the samples were prepared as detailed above. The optical density in each case was colorimetrically recorded as above. The amount of quercetin, luteolin and kaempferol in the samples were then determined (mg/100g.d.w.) by comparing with those of their respective standard regression curves. Five such replicates were examined and mean values were calculated (SE < 0.5 %).

## 3. Results and Discussion

Presence of Quercetin (Rf 0.82, m.p.309-311$^0$ C,UV max 258, 373, yellowish blue with $FeCl_3$ ) Luteolin ( Rf 0.78, m.p.328$^0$C, UV max 255,350, green with $FeCl_3$) and Kaempferol (Rf 0.93, m.p. 271-273$^0$ C, UV max 268, 368 deep yellow to brown with $FeCl_3$) have been identified, confirmed and measured quantitatively in all samples of unorganized cultures of ***A. excelsa*** while Quercetin and Kaempferol in ***B. eagyptiaca.*** The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of quercetin, luteolin and kaempferol in all samples.

Maximum GI was observed at the age of eight weeks in standardized (Sd) MS medium and standaized MS media supplemented with various concentrations ( 1,2,3 mg/L ) of growth hormones ( IAA and NAA ) and salts (NaCl and KCl ( 10,20,30 mg/L ) in ***A. excelsa*** and ***B. eagyptiaca.*** Calli were harvested at maximum GI from all the samples separately in both plants.

It was observed that amount of flavonoids was increased in callus fed with growth regulators IAA and NAA. Increase was continuous from Sd MS medium to Sd MS medium fed with 1mg/L and then started decreasing continuously upto 3 mg /L IAA and NAA separately in both plant species. The amount calculated in calli fed with 2 and 3mg/L IAA and NAA was lower than amount of flanonoids present in calli grown on Sd MS medium. Maximum amount of flavonoids was calculated in calli fed with 1mg/L IAA (0.47+0.40+0.51=1.38 mg/100 g.d.w. and 0.45+0.48=0.93 mg/100 g.d.w. respectively ) and NAA(0.48+0.40+0.52=1.40 mg/100 g.d.w. and 0.44+0.48=0.92 mg /100 g.d.w. respectively) in ***A. excelsa*** as well as ***B. eagyptiaca*** ( Table 1).

In calli fed with salts KCl and NaCl , the amount of flavonoids was increased from Sd MS medium to calli fed with 10 mg/L and then decreased from 10 mg/L to 20 mg/L upto 30 mg/L in both plant species. Maximum amount was calculated in calli fed with 10mg/L NaCl (0.49+0.41+0.52=1.42 mg/L and 0.46+0.49=0.95 mg/L respectively )and KCl (0.48+0.40+52=1.40 mg/L and 0.46+0.48=0.94 mg/L respectively) in ***A. excelsa*** as well as ***B. eagyptiaca*** ( Table 2 ).

Amount of quercetin is higher than kaemferol in all the samples of both plant species and growth hormones have been proved to be better for increasing the flavonoid content. Luteolin is present only in ***A. excelsa*** and its amount is lowest.

## 4. Conclusion

Up to acertain concentration of growth regulators, the amount of flavonoids can be increased in vitro and it can be useful on large scale production.

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**Table 1:** Effect of Growth Regulators On Flavonoid Content (mg/100 g.d.w.) IN *A. excelsa* AND *B. aegyptiaca IN Vitro* (At Maximum GI)

| Name of Plant | Flavonoids | Sd MS Medium | Salts | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | IAA/L | | | NAA/L | | |
| | | | 1mg | 2mg | 3mg | 1mg | 2mg | 3mg |
| *A. excelsa* | Kaempferol | 0.46±0.05 | 0.47±0.06 | 0.45±0.06 | 0.40±0.04 | 0.48±0.04 | 0.44±0.05 | 0.41±0.06 |
| | Luteolin | 0.38±0.04 | 0.40±0.05 | 0.35±0.03 | 0.31±0.06 | 0.40±0.04 | 0.37±0.03 | 0.30±0.03 |
| | Quercetin | 0.50±0.06 | 0.51±0.06 | 0.48±0.07 | 0.45±0.05 | 0.52±0.04 | 0.49±0.03 | 0.43±0.05 |
| | Total | 1.34±0.05 | 1.38±0.05 | 1.28±0.06 | 1.16±0.07 | 1.40±0.06 | 1.30±0.04 | 1.14±0.05 |
| *B.aegyptiaca* | Kaemphferol | 0.42±0.06 | 0.45±0.07 | 0.42±0.05 | 0.36±0.05 | 0.44±0.05 | 0.40±0.06 | 0.34±0.04 |
| | Quercetin | 0.46±0.04 | 0.48±0.04 | 0.45±0.05 | 0.39±0.06 | 0.48±0.06 | 0.44±0.04 | 0.37±0.05 |
| | Total | 0.88±0.06 | 0.93±0.04 | 0.87±0.04 | 0.75±0.05 | 0.92±0.05 | 0.84±0.04 | 0.71±0.05 |

Values are mean of five replicates ± SD

**Table 2:** Effect of Salts on Flavonoid Content (mg/100 g.d.w.) IN *A. excelsa* AND *B. aegyptiaca IN VITRO* (At Maximum GI)

| Name of Plant | Flavonoids | Sd MS Medium | Salts | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | NaCl/L | | | KCl/L | | |
| | | | 10mg | 20mg | 30mg | 10mg | 20mg | 30mg |
| *A. excelsa* | Kaempferol | 0.46±0.04 | 0.49±0.04 | 0.44±0.06 | 0.40±0.04 | 0.48±0.05 | 0.44±0.08 | 0.38±0.06 |
| | Luteolin | 0.38±0.06 | 0.41±0.07 | 0.37±0.06 | 0.33±0.04 | 0.40±0.06 | 0.36±0.06 | 0.30±0.04 |
| | Quercetin | 0.50±0.08 | 0.52±0.05 | 0.49±0.07 | 0.46±0.05 | 0.52±0.03 | 0.48±0.06 | 0.45±0.08 |
| | Total | 1.34±0.05 | 1.42±0.05 | 1.30±0.06 | 1.19±0.04 | 1.40±0.05 | 1.28±0.06 | 1.13±0.04 |
| *B.aegyptiaca* | Kaempferol | 0.42±0.04 | 0.46±0.06 | 0.43±0.04 | 0.38±0.05 | 0.46±0.04 | 0.42±0.04 | 0.35±0.05 |
| | Quercetin | 0.46±0.07 | 0.49±0.06 | 0.46±0.03 | 0.40±0.02 | 0.48±0.03 | 0.45±0.05 | 0.38±0.05 |
| | Total | 0.88±0.06 | 0.95±0.05 | 0.89±0.02 | 0.78±0.03 | 0.94±0.02 | 0.87±0.04 | 0.73±0.05 |

Values are mean of five replicates ± SD