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Identification, Isolation and Estimation of Flavonoids and Effect of Growth Regulators and Salts on Flavonoids in *Aegle marmelos* and *Moringa oleifera in vitro*

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Abstract: ***Unorganized tissues of medicinally useful plants Aegle marmelos and Moringa oleifera were established on MS medium supplemented with 1mg/L BAP+2mg/L 2,4-D and 1.5mg/L BAP +1.5mg/L 2,4-D respectively (standardized MS medium). Parts of established tissues were transferred to standardized (Sd) MS medium fed with various concentrations(1,3,5 mg/L) of growth hormones (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 2mg/L IAA, NAA and 10mg/L NaCl and KCl in A. marmelos as wll as M. oleifera .***

Keywords: Aegle marmelos, Moringa oleifera, flavonoids, growth regulators, salts

1. Introduction

Aegle marmelos* Corr.** commonly known as "Bael" is medium-sized slow growing deciduous spiny woody fruit tree of the tropics. It belongs to the family Rutaceae**.** Bael is a sacred tree, dedicated to Lord Shiva. It is extensively planted near Hindu temples for its leaves and wood which are used for worship and for its edible fruits which are valued in indigenous medicine. Pharmacologically ***Aegle marmelos is having antibacterial, antihistaminic, antiinflammatory, anticonvulsant, antistress and adaptogenic, antipyretic, antifertility, analgesic, hepatoprotective, insecticidal, hypoglycemic, immunomodulatory, testicular, cardiotonic, wound healing activity.

According to the Ayurveda, ***Moringa oleifera*** (family-Moringaceae) is commonly called 'Sigru'. Various parts of the ***Moringa*** tree such as root, root bark, leaves flowers, unripe pods, seeds, seed oil are used in Ayurvedic system of medicine. Sigru (***Moringa oleifera***) is used externally as well as internally. Externally, the paste of its leaves and bark skin is applied in boils to subside the swelling and inflammation. Internally, Sigru is used in vast range of diseases. The pods are recommended in loss of appetite. It is also beneficial in treatment of worms, anorexia, ascites, tumors, abdominal pain, paralysis, amenorrhea, dysmenorrhea, joint pain and gout.

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.

Flavonoids are 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 hypogeal*** (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).

2. Materials and Methods

Unorganized tissue of ***A. marmelos*** and ***M. oleifera*** were established on MS medium supplemented with 1mg/L BAP+2mg/L 2,4-D and 1.5mg/L BAP +1.5mg/L 2,4-D respectively (standarized media for both species). Parts of established tissues were transferred to standardized (Sd) MS medium fed with various concentrations(1,3,5 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, all variations of growth regulators and salts in both plant species) were harvested, dried, powdered and analyzed for estimation of flavonoids.

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.

Qualitative Analysis

Thin glass plates coated with silica gel G were dried, activated at 100^0C 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 one fluorescent spot in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third) in all the samples of ***A. marmelos*** and ***M. oleifera*** coinciding with those of the standard samples of quercetin (yellow, R_f 0.82) and 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, kaempferol–deep yellow). On spraying the developed plates with 5% ethanolic ferric chloride solution one spot was seen in ethyl ether fraction (second) and one spot in ethyl acetate fraction (third)**.** Fraction second spots were coinciding with those of reference quercetin (yellowish brown) and that of fraction third with kaempferol (deep yellow) in all samples. The R_f values were calculated as an average of the five replicates.

Preparative Thin Layer Chromatography (PTLC)

Glass plates thickly coated with silica gel G were used for preparative thin layer chromatography (PTLC). The extract of both the fractions (second and third) of ***A. marmelos*** and ***M. oleifera*** 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 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.

Quantitative Analysis

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 and of **Mabry** *et al.,* **(1970)** in case of kaempferol.

3. Results and Discussion

Presence of Quercetin (Rf 0.82, m.p. 309-311^{0}C, UV max 258, 373, yellowish blue with $FeCl_3$) 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. marmelos*** and ***M. oleifera.*** The characteristic IR spectral peaks were coinciding with those of their respective standard reference compounds of quercetin 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 at 10,20,30 mg/L) in ***A. marmelos*** and ***M. oleifera*** . 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 up to Sd MS medium fed with 2 mg /L IAA and NAA but after that amount decreased from 2mg/L to 3mg/L IAA and NAA separately in both plant species. The amount calculated in calli fed with 3mg/L IAA and NAA was lower than amount of flanonoids present in callus grown on Sd MS medium. Maximum amount of flavonoids was calculated in callus fed with 2mg/L IAA and NAA in ***A. marmelos*** as wll as ***M. oleifera***

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 and KCl in ***A. marmelos*** as wll as ***M. oleifera*** . 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.

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. marmelos* and *M. oleifera IN VITRO* (at Maximum GI)

Name of Plant	Flavonoids	Sd MS Medium	Salts					
			IAA/L			NAA/L		
			1mg	2mg	3mg	1mg	2mg	3mg
A.marmelos	Quercetin	0.67±0.05	0.71±0.06	0.65±0.06	0.68±0.04	0.70±0.04	0.70±0.05	0.63±0.06
	Kaempferol	0.60±0.06	0.64±0.05	0.69±0.07	0.62±0.05	0.62±0.04	0.66±0.03	0.56±0.05
	Total	1.30±0.05	1.35±0.05	1.45±0.06	1.32±0.05	132±0.04	1.39±0.03	1.31±0.05
M. oleifera	Quercetin	0.58±0.06	0.64±0.05	0.68±0.04	0.62±0.05	0.60±0.05	0.64±0.06	0.59±0.07
	Kaempferol	0.55±0.07	0.60±0.04	0.64±0.05	0.59±0.06	0.58±0.06	0.61±0.04	0.56±0.05
	Total	1.13±0.06	1.24±0.04	1.32±0.04	1.21±0.05	1.18±0.05	1.25±0.04	1.15±0.05

Values are mean of five replicates ± SD

Table 2: Effect of Salts on Flavonoid Content (mg/100g.d.w.) in *A. marmelos* and *M. oleifera IN VITRO* (At Maximum GI)

Name of Plant	Flavonoids	Sd MS Medium	Salts					
			NaCl/L			KCl/L		
			10mg	20mg	30mg	10mg	20mg	30mg
A.marmelos	Quercetin	0.67±0.05	0.68±0.04	0.66±0.06	0.61±0.04	0.69±0.05	0.66±0.08	0.63±0.06
	Kaempferol	0.60±0.06	0.61±0.05	0.58±0.06	0.53±0.04	0.62±0.03	0.58±0.06	0.55±0.04
	Total	1.27±0.05	1.29±0.05	1.24±0.06	1.14±0.04	1.31±0.05	1.24±0.06	1.18±0.04
M. oleifera	Quercetin	0.58±0.06	0.60±0.05	0.57±0.02	0.52±0.03	0.61±0.02	0.58±0.04	0.54±0.05
	Kaempferol	0.55±0.07	0.56±0.06	0.53±0.03	0.50±0.02	0.57±0.03	0.54±0.04	0.51±0.05
	Total	1.13±0.06	1.16±0.05	1.10±0.02	1.02±0.02	1.18±0.02	1.12±0.04	1.05±0.05

Values are mean of five replicates ± SD