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A Comparative Study of Secondary Metabolites, Amino acids and Protein Profiles of the Host – Parasite Plants in the Relationship between the African Mistletoe, *Tapinanthus bangwensis* [Engl. and K. Krause] Danser and Two of its Host species

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ABSTRACT

The presence of secondary metabolites, amino acids and sugar alcohol including protein profile in the host-parasite relations of *Tapinanthus bangwensis* (*Tb*) on the host plants, C*itrus sinensis* (*Cs*) and *Irvingia gabonensis* (*Ig*) were evaluated. Secondary metabolites contained in the host-parasite plants was observed for the *Citrus*-parasite (tannin - 0.35 *Tb*, 0.21 *Cs*; phenol - 0.21 *Tb*, 0.14 *Cs*; alkaloids - 1.42 *Tb*, 0.17 *Cs* g/100g) and *Irvingia*-parasite (tannin - 0.23 *Tb*, 0.18 *Ig*; phenol - 0.19 *Tb*, 0.15 *Ig*; alkaloids - 1.34 *Tb*, 0.57 *Ig* g/100g) with comparative higher quantities in the parasite. Free amino acids and sugar alcohol contents in the leaves of host plants revealed the constituents of some of the groups in the infested (tyrosine - 12.26 *Cs,* 14.70 *Ig*; aspartic acid - 12.21 *Cs,* 11.23 *Ig* ng/g) and uninfested (tyrosine - 10.76 *Cs,*12.93 *Ig*; aspartic acid - 9.09 *Cs,* 9.79 *Ig* ng/g) which indicated significant higher values for the infested. Protein profiling of the C*itrus* leaves revealed lack of protein at 25.0 kDa band in the infested host. Assessment of metabolites and protein features in the mistletoe-host relationship affirmed presence of similar metabolites in the host-parasite entity while infested hosts had increased free amino acids and there were noticeable variations in protein banding pattern of host plants with mistletoe incidence.

INTRODUCTION

Mistletoes are generally parasites which have form an intimate physiological connection with their hosts often leading to change and or adaptive variation in phenotype (and usually as well genetic change) for them and their host plants. Mistletoe plants may become so intrinsically connected to the host that it may seem like another branch (in cases with strong mimicry); but mistletoe on host is separate and distinct with its metabolic process and photosynthetic interaction for sustenance. The various groups of mistletoes in interaction with their host gave rise to different display of physiological dependence on host photosynthesis. The hemiparasite autotrophs rely on water and inorganic nutrients supply of the host through their (host) xylem while the holoparasites, in addition to depending on water and inorganic solutes of their host make use of the photosynthate of their host (Glatzel and Geils, 2009). Mistletoe–host compatibility is a function of host susceptibility to infection and of mistletoe infectivity (Yan, 1993). If mistletoes are physiologically, biochemically and physically compatible with a tree, they will have a chance to germinate, establish and survive on that tree (Roxburgh and Nilcolson, 2005).

Most genera of African mistletoes belong to the family Loranthaceae (Polhill and Wiens, 1998). In West Africa and Nigeria in particular, mistletoes are found on many agronomic tree crops which include the shea butter tree (*Vitellaria paradoxa* Gaertn. f.), *Citrus* species; mostly sweet orange (*Citrus sinensis* L.) and grape (*Citrus paradisi* L.), and cocoa (*Theobroma cacao* L.). Different species of these hemi-parasitic plants grow on other medicinal as well as cultivated trees such as the brimstone tree (*Morinda lucida* Benth), the kola-nut tree (*Cola nitida* Vent. Schot and Endl.), forest trees such as *Irvingia gabonensis* (Aubrey-Lecomte ex O. Rorke) Baill, *Parkia biglobosa*, among a host of other tree crops (Wahab *et al*., 2010; Adesina *et al*., 2013; Ibrahim *et al*., 2014).

Mistletoes are used severally in traditional medicine practice. The group, especially of the Loranthaceae and Viscaceae are widely used by different races and cultures in almost every continent to treat various ailments including hypertension and diabetes, or used as a diuretic agent (Adesina *et al*., 2013). The plant is ethnomedicinally used by the different ethnic groups in Nigeria as a remedy for several human and animal ailments such as dysentery, diarrhoea, convulsion (Ilesanmi and Olawoye, 2011), cardiovascular diseases and gynaecology problems (Adodo, 2004). It has been found to have anti-microbial properties against certain multiple-drug-resistant bacterial and fungal isolates of farm animals (Deeni and Sadiq, 2002).

There had been observed variation in secondary metabolites among same mistletoe species occurring on different host plants (Wahab *et al*., 2010; Ilesanmi and Olawoye 2011; Umoh *et al*., 2011; Ibrahim *et al*., 2014). The screening for phytochemical substances in various species of the African mistletoes reflects divergent and varying constituent of the Alkaloids, Saponin, Tannin, Phlobatanin, Anthraquinone, Cardiac glycosides, Cardenolides, Steroidal nucleus, reducing sugar, flavonoids among others in their composite formation.

It has been suggested that pharmacologically active compounds may pass from the host trees to the parasitic plants. Thus, biological activities of the parasitic plant could differ, just as the apoptosis-inducing properties of *Viscum album* extract has been found to be host dependent (Bussing and Schietzel, 1999). The influence of the host chemistry on the chemical constituents of the parasite on different hosts might justify why the host is as important as the parasite in pharmacognosy, ethnopharmacology and ethnomedicine, and why the use of these mistletoes in the treatment of an ailment is often dependent on a particular or specific host (Burkill, 1995; Snyder *et al*., 1996; Adodo, 2002; Olapade, 2002; Preston *et al*., 2010).

Parasitic plants often connect to their host through a continuous vascular system (and plasmodesmata) in some species like *Striga* and *Cuscuta.* This linkage has been reported served in part for movement of molecules between these plants (Ichihashi *et al*., 2015). Macromolecules are naturally transported into the parasite from the host plant, and several reports have implicated haustorial connections in DNA, RNA, protein and also viruses translocation between host and parasite (Mower *et al*., 2004; Roney *et al*., 2007; David-Schwartz *et al*., 2008; Ichihashi *et al*., 2015).

Plant reactions and responses to either biotic or abiotic stress conditions is a complex phenomenon involving alterations in physiological and biochemical processes, which may result in morphological and developmental changes (Azevedo Neto *et al*., 2009). In plants, most of the observed stress conditions are generally correlated with enhanced proteolytic activity and increased protein turnover, leading to either acclimation to the stress condition or to senescence and subsequent cell death (Martinelli *et al.*, 2007). Several investigations have shown increased accumulation of free amino acids especially proline, during adaptation to various environmental stresses (Simon-Sarkadi *et al*., 2002; Kaplan *et al.*, 2004; Zuther *et al*., 2007; Kempa *et al*.,2008; Sanchez *et al*., 2008; Usadel *et al*., 2008; Lugan *et al*., 2010; Murugan *et al*., 2014).

The accumulation of soluble sugars and sugar alcohols (e.g. pinitol) is known to be another common metabolic response of higher plants to stress. Pinitol is a common sugar alcohol present in a wide range of plant species including legumes and some other higher plants. A handful of the research work on plants under stress

conditions of high temperature, salinity and drought, so far revealed the potential physiological acclimation of such plants through a means of increase in pinitol accumulation (Murakeözy *et al*., 2002; Nayyar 2003; Griffin *et al*., 2004; Li-Xia *et al*., 2008).

Considering the extent of work done on African mistletoes, there are few staggered investigations on presence of secondary metabolites in this group of parasites with even scanty efforts on amino acids and protein profiles of the parasites; especially with respect to their host plants. Most of the available works have not extensively addressed the host-parasite relationship along the path of products of their secondary metabolites, amino acids and protein profiles. Therefore, this research was undertaken to investigate the likelihood of correlation in the output of secondary metabolites from mistletoe and its host plants; and to also determine if the presence of mistletoe has influence on the amino acids and protein profiles of the host species.

MATERIALS AND METHODS

Site of the study

Stems and leaves of mistletoe on *Citrus* and *Irvingia* as well as same from host plants were collected, cleaned and dried in the sun. The dried materials were ground to powder and preserved in air tight containers for the necessary analyses. The samples were collected from plantation fields at Moor Plantation, Apata, Ibadan, South-Western, Nigeria (located at latitude, $07^0$3872' - $07^0$3860'N; longitude, $003^0$8420' - 003^0 8415'E; and at an altitude of 3 m). The laboratory works were conducted at the Central Laboratory / Biotechnology Centre, Federal University of Agriculture, Abeokuta (FUAAB) and the Institute of Agricultural Research and Training (IAR&T).

Phytochemical Screening of Secondary Metabolites

Qualitative Analysis

Test for the presence of tannins, phenols, saponins, alkaloids, flavonoids, oxalate, phytate, terpenes, and steroids content of the plant were determined by the methods described by Trease and Evans (1989), Early and De Turk (1944) and Sofowora (1982).

Quantitative Analysis

Determination of Tannic Acid (Tannin)

One gramme (1g) of each sample of the mistletoe and host plants was weighed into a beaker. A solvent mixture containing 80 ml acetone and 20 ml glacial acetic acid was used to extract tannin by soaking each sample for 5 hours. Filter paper with double layer used to filter the samples and the filtrates obtained were then removed. A set of standard solution for tannic acid ranging from 10 ppm to 50 ppm was prepared. With the use of spectronic 20, absorbances of the standard solution and filtrates were read at 500 nm wavelength.
Percentage tannin was obtained with the calculation below:

$$\% \text{ Tannin} = \frac{\text{Absorbance X Average gradient X Dilution factor}}{10,\ 000}$$

Determination of Total Phenol

One gramme (1g) of each sample of the mistletoe and respective (infested) host plants was weighed and transferred into 250 millilitres conical flask. Twenty millilitres (20 ml) volume of distilled water was used in soaking each sample. These samples were filtered after 4 days and each filtrate measured was marked up with distilled water to 100 ml in volumetric flask. A (one) 1 ml volume of the filtrate from each sample was transferred into a test tube and 3 ml each of 0.01 N Iron (III) chloride and 0.008 N Potassium hexacyanoferrate (III) were added into each filtrate. Absorbance of the filtrate from each sample was noted after 10 minutes at 760 nm. Total polyphenol percentage was measured as below:

$$\% \text{ Total polyphenol} = \frac{\text{Absorbance X Average gradient X Dilution factor}}{\text{Weight of sample X } 10,\ 000}$$

Determination of Saponin

Twenty grammes (20 g) each of the samples of mistletoe and host plants was weighed and transferred into a conical flask and 100 ml of 20% isobutylalcohol (octanol) was added thus heated over a hot water bath for 4 h at about 55°C with continuous stir for thorough mixing in order to obtain a uniform solution. Filtration was then performed on the mixture and the residue re-extracted using another 200 ml 20% ethanol. Reduction of the combined extracts to 40 ml over a water bath was undertaken at about 90°C. The concentrate was transferred into a 250 ml separatory funnel and 20 ml of diethyl ether was added and shaken vigorously. The ether layer was discarded while the aqueous layer was recovered and a repeat of the purification process was done. 60 ml of n – butanol was added and the combined n – butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was then heated in a water bath after evaporation; the

samples were dried in the oven to a constant weight. Percentage saponin was obtained with the calculation below:

$$\text{Saponin in \%} = \frac{\text{Residue weight}}{\text{Sample weight taken}} \times 100$$

Determination of Total Alkaloids

Five gramme (5 g) each of the samples of mistletoe and host plants (stems and leaves) was weighed and transferred into beaker of 250 ml volumetric capacity into which was added 200ml of 10% solvent of acetic acid in ethanol. The beaker containing the mixture was covered (to check evaporations of solvent) and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath to one – quarter of the original volume. The whole solution was allowed to settle and the precipitate was collected and washed using dilute ammonium hydroxide which was ultimately subjected to filtration. The precipitate residue which was dried and weighed is the alkaloid.

$$\text{Weight of total alkaloids: } \frac{W2 - W1}{W3} \text{ g,}$$

$$\text{\% Yields of Alkaloid: } \frac{W2 - W1}{W3} \times 100$$

Where, W1 = crucible weight, W2 = crucible and alkaloids weight, W3 = plant sample initial weight taken for estimation.

Determination of Flavonoids

One gramme of the powdered stem and leaf samples of the mistletoe and infested host plants (*Citrus* and *Irvingia*) was weighed into 250 ml flask. Warm distilled water of 20 ml was added to the sample and placed inside water bath for 10 minutes at 100^0C. Subsequently, filtration was done. With the use of a pipette, 1 ml of the filtrate was collected and emptied into a clean test-tube while 1 ml of 0.5 N NaOH was added. Distilled water of about 8 ml was added and allowed to stand for 10 mins. Using spectronic 21D, absorbance was measured at 410 nm wavelength. The standards were prepared using 2.5 ppm, 1.5 ppm, 1 ppm and 0.5 ppm.

Calculation: absorbance x average gradient x dilution factor

Determination of Oxalate

Extract of samples (obtained from powdered stem and leaf) of the mistletoe (*Tapinanthus bangwensis*) and host plants (*Citrus sinensis* and *Irvingia gabonensis)* each weighing one gramme was placed into 250 ml conical flask soaked in 100 ml distilled water. The soaked samples after 3 hours were each filtered through a filter paper of double layer. A standard solution of oxalic acid was prepared which comprised 10 ppm, 20 ppm, 30 ppm, 40 ppm and 50 ppm. The absorbance was then measured on spectrophotometer at 420 nm wavelength. Absorbance of filtrate obtained from each sample was also measured on the spectronic 20. The percentage oxalate was obtained using the expression:

$$\text{\% oxalate} = \frac{\text{sample absorbance X average gradient from the curve for standard X dilution factor}}{10,000}$$

Determination of Phytic Acid (Phytate)

Two (2) grammes of the sample of each plant materials (the mistletoe and associated host plants which include the *Citrus* and *Irvingia*) was weighed and transferred into 250 ml conical flask. Each sample soaked in 100 ml volume of 2% conc. Hcl poured into a conical flask was left for 3 hours after which time a hardened filter paper with double layer was used for filtration. With 250 ml beaker into which has been poured 50 ml of each filtrate a 107 ml volume distilled water was added in each case for proper acidity. To each solution was added an indicator containing 10 ml solution of 0.3% ammonium thiocynate. With the use of standard Iron (III) chloride solution containing 0.0019 g iron per millilitre, the solution was titrated. The slightly brownish – yellow end point which persisted for about 5 minutes was the positive indicator. Percentage phytic acid was obtained with the calculation below:

$$\text{\% Phytic acid} = \frac{X \times 1.19 \times 100}{2}$$

Where X = Titre value X 0.00195

Determination of Terpene

With a 50 ml conical flask in place and weighing 0.50 g therein (powdered stem and leaf samples each obtained from the mistletoe and attached hosts), 20 ml volume of 2:1 chloroform-methanol mixture was added and thoroughly shaken before allowing the whole mixture to stand for 15 minutes. The mixture was subsequently centrifuged for yet another 15 minutes. Hereupon, another 20 ml chloroform-methanol mixture was used to rewash the precipitate got for re-centrifugation while the supernatant obtained was discarded.

A solution of 40 ml volume of 10% Sodium deodocyl sulphate was used to dissolve the resultant precipitate. One millilitre (1 ml) of 0.01M Ferric chloride solution was added to the above at 30 seconds interval shaken well and allowed to stand for 30 minutes. Using a stock of 100 mg/l terpenes solution, concentration range of 0-5 mg/ml standard terpenes was prepared. Absorbances for sample as well as standard concentrations of terpenes were read on a Digital Spectrophotometer at a

wavelength of 510 nm. Percentage terpene was obtained with the calculation below:

$$\frac{\text{Absorbance of sample X dilution factor X gradient factor}}{\text{Wt. of sample X 10, 000}}$$

Determination of Steriods

A Half gramme (0.50 g) extract, each obtained from powdered sample (stem and leaf) of *Tapinanthus* and the infested hosts, *Citrus* and *Irvingia* was weighed and transferred into a 100 ml beaker. To dissolve the extract, a mixture of 20 ml chloroform-methanol (2:1) was added; then placed on a shaker and shaken for 30 minutes. The whole mixture was then filtered through a Whatman No. 1 filter paper into another 100 ml conical flask that was clean and dry.

Resultant residue was rendered steroids-free by repeated treatment with chloroform-methanol mixture. To achieving a homogenous mixture, 1 ml of filtrate got with a pipette was poured into a 30 ml test tube then 5 ml volume of alcoholic KOH was further added and thoroughly shaken. The mixture was subsequently set in a water bath which had been fixed at 37^0C – 40^0C for 90 minutes, afterwards allowed to cool to ambient temperature and 10 ml Petroleum ether added with further 5 ml of distilled water also added. This mixture left in a water bath was heated to dryness. To the residue in dry bottle was added 6 ml Liebermann Burchard reagent and absorbance taken at wavelength of 620nm on Spectronic 21D digital spectrophotometer.

Prepared from 100 mg/ml stock of standard steroids solution were standard steroids of concentration range between 0-4 mg/ml and treated similarly like sample as above.
% Steroid was calculated as below:

$$\frac{\text{Absorbance of sample X Gradient X Dilution Factor}}{\text{Wt of sample X 10000}}$$

Determination of Trypsin Inhibitor

A sample (powdered stem and leaf) of 0.2g of the mistletoe and host plants was transferred into a screw cap centrifuge tube with 10 ml 0.1M phosphate buffer addition and the content was shaken at room temperature on a UDY shaker for 1 hour. The suspension obtained was filtered through whatman No. 42 filter paper after centrifugation at 5000 rpm for 5 mins. With phosphate buffer, the volume of each filtrate was adjusted to 2ml. The test tubes were placed in water bath, maintained at 37^oC. Six millilitres (6ml) of 5% TCA solution was added to one of the tubes previously kept at 37^oC. These were incubated for 20 mins. The reaction process was stopped after 20 mins by adding 6ml of TCA solution to the experimental tubes and shaken. The reaction was allowed to proceed for 1 hour at room temperature. Filtration of the mixture obtained was carried out using Whatman No. 42 filter paper. The filtrate from sample and trypsin solutions was read at 280 nm for their absorbance. The trypsin inhibitor measured in mg/g sample was obtained as below:

$$\text{T. I. mg/g} = \frac{\text{A std - A sample}}{\text{0.19 X sample wt in g}} \times \frac{\text{Dilution factor}}{\text{1000 X sample size}}$$

Determination of Free Amino Acids

The ninhydrin colorimetric analysis method of Rosen (1957) was adopted for the (free) amino acids test. Extracts were got from leaf samples of *Citrus sinensis* and *Irvingia gabonensis* with each diluted as appropriate. To 1 ml of the diluted leaf extract in methyl cellosolve were added 0.5 ml of 3% ninhydrin and 0.5 ml Cyanide acetate buffer. Using water bath, the mixture was heated for 15 mins at 100^0C. A further addition of 5 ml isopropyl alcohol water mixture with vigorous shaking was soon carried out. The colour of the mixture was read in colorimeter at 570 nm after it had been left to cool. Based on the known concentration of various amino acids, concentrations of the amino acids were calculated and obtained from a standard graph.

SDS-Page Test for Extract of Soluble Proteins from Leaf Specimens

800µl. 0.1M Tris-Hcl of pH 7.6 was added to 0.3g of the leaf specimen (obtained from the infested and uninfested host plants of *Citrus* and *Irvingia*) ground to powder which was subsequently subjected to vortex for 1 min; then spun down at the speed of 10, 000 rpm for 5 minutes. Resultant supernatant collected was placed in a new Enpendorff tube and kept at 4^oC. The gel was prepared at 12% separating gel and 4% stack gel; the test material and the standard were thus loaded for the gel to be run at 150v for 45 minutes. The resultant product was stained in commassie blue for 45 minutes and later de-stained with de-staining solution with several changes until bands showed clearly.

Statistical Analysis

Data obtained was considered statistically by using the SPSS 21 Statistics Program. Statistical analysis was done using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT).

RESULTS

Table I showed the status of secondary metabolites as obtained in the mistletoe (*Tapinanthus bangwensis*) in relation to its two hosts; *Citrus sinensis* and *Irvingia gabonensis.* Tests for tannin indicated strongly positive presence (+++) in the mistletoe on *Citrus* while the host plant had positive (++) outlook. Indication of presence of

tannin in the *Irvingia*–mistletoe association was positive in the host while it was strongly positive in the parasite. Phenol presence in the mistletoe and the two hosts (*Citrus* and *Irvingia*) was positive. Examination for saponin in both mistletoe and its *Citrus* host yielded positive presence. Also saponin in the mistletoe on *Irvingia* was positive (++) compared to the trace (+) indication of saponin presence in the *Irvingia*. Alkaloid was strongly positive (+++) for the mistletoe on *Citrus* in comparison to its positive indication (++) in the host. However, alkaloids presence was positive for both the mistletoe on *Irvingia* and the host. The mistletoe on *Citrus* and the host manifested positive presence of flavonoid. The presence of flavonoid observed in the mistletoe on *Irvingia* was positive (++) but trace (+) for the host plant. The mistletoe on *Citrus* and *Irvingia* together with the two hosts gave indications of positive presence of oxalate. Phytate presence in the mistletoe on *Citrus* and *Irvingia* and the two hosts as well was positive. It was a reflection of trace presence of terpenes in the mistletoe on *Citrus* and the host but the mistletoe on *Irvingia* showed trace presence compared to the positive presence observed in its host. Trace presence of steroids was observed in the mistletoe on *Citrus* while steroid presence was positive in the host. The *Irvingia*–mistletoe association reflected trace presence of steroids for the parasite and host.

TABLE I: Secondary Metabolites presence in *Tapinanthus bangwensis* and its two hosts

Sample	Tannin	Phenol	Saponin	Alkaloids	Flavonoids	Oxalate	Phytate	Terpenes	Steroids
T. bangwensis on *Citrus sinensis*	+++	++	++	+++	++	++	++	+	+
Citrus sinensis	++	++	++	++	++	++	++	+	++
T. bangwensis on *Irvingia gabonensis*	+++	++	++	++	++	++	++	+	+
Irvingia gabonensis	++	++	+	++	+	++	++	++	+

KEY: + = TRACE; ++ = POSITIVE; +++ = STRONGLY POSITIVE

The quantitative array of secondary metabolites present per each host species in relation to its attached mistletoe is shown in Table II. The observed amount of tannin in the mistletoe (0.35 g/100g) on *Citrus* was significantly higher ($\alpha = 0.05$) than the amount obtained in the host. The mistletoe on *Irvingia* also had higher tannin content than its host. The phenol content of *Tapinanthus* on the *Citrus* and *Irvingia* hosts was higher than the observed content in each host. Saponin in the mistletoe on *Citrus* and that of its host was not statistically different but the mistletoe on *Irvingia* had significantly higher amount than what was available in the host. The alkaloid in the mistletoe (1.42 g/100g) on *Citrus* was significantly higher ($\alpha = 0.05$) than that in the host and similarly the mistletoe on *Irvingia* had higher alkaloid content than the host. Flavonoid in the mistletoe (1.45 g/100g) on *Citrus* was significantly higher than that of the host but the *Irvingia* host plant however had significantly higher quantity (0.31 g/100g) of flavonoid than its parasite. The quantity of the oxalate in *Citrus* host was significantly higher than its parasite while the parasite on *Irvingia* had higher amount than in its host. Phytate in the mistletoe (0.41 g/100g) on *Citrus* was significantly higher than that in the host and the phytate content in the mistletoe (0.52 g/100g) on *Irvingia* was also higher than in its host. The observed amount of terpenes present in the mistletoe on the *Citrus* and *Irvingia* hosts was significantly higher than the respective host. Steroid obtained in the mistletoe on *Citrus* and *Irvingia* hosts was in higher amounts than that available in the hosts. Trypsin inhibitor content of the mistletoe and both *Citrus* and *Irvingia* hosts were statistically similar.

TABLE II: Secondary Metabolites contents in *Tapinanthus bangwensis* and its two hosts

Sample	Tannin g/100g	Phenol g/100g	Saponin g/100g	Alkaloids g/100g	Flavonoids g/100g	Oxalate g/100g	Phytate g/100g	Terpenes g/100g	Steroids g/100g	Trypsin inhibitor g/100g
T. bangwensis on *Citrus sinensis*	0.35^{a}	0.21^{a}	0.18^{c}	1.42^{a}	1.45^{a}	0.50^{c}	0.41^{b}	0.08^{b}	0.11^{a}	0.13^{a}
Citrus sinensis	0.21^{c}	0.14^{d}	0.19^{b}	0.17^{d}	0.22^{c}	1.24^{a}	0.12^{d}	0.02^{d}	0.01^{c}	0.13^{a}
T. bangwensis on *Irvingia gabonensis*	0.23^{b}	0.19^{b}	0.80^{a}	1.34^{b}	0.18^{d}	0.62^{b}	0.52^{a}	0.09^{a}	0.02^{b}	0.13^{a}
Irvingia gabonensis	0.18^{d}	0.15^{c}	0.19^{b}	0.57^{c}	0.31^{b}	0.45^{d}	0.33^{c}	0.03^{c}	0.01^{c}	0.11^{b}

Means followed by the same letter in each column are not significantly different by DMRT at $\alpha = 0.05$

Shown in Table III were the results on content of the free amino acids and stress metabolites in the leaf specimens of infested and uninfested *Citrus* and *Irvingia* host plants. Tyrosine in the infested leaf of *Citrus* (12.26 ng/g) was significantly higher (α = 0.05) than the uninfested. And the infested leaf of *Irvingia* (14.70 ng/g) also possessed significantly higher quantity of tyrosine than the uninfested. Phenylalanine in the uninfested *Citrus* (14.15 ng/g) was significantly higher than the infested but the infested leaf of *Irvingia* (16.97 ng/g) had significantly higher phenylalanine content than the uninfested. The serine assessment showed that the infested leaves of the *Citrus* (11.49 ng/g) and *Irvingia* (13.49 ng/g) hosts had higher contents of the amino acid than the uninfested. Glycine in the *Citrus* and *Irvingia* hosts showed contrasting outcomes as the uninfested *Citrus* (10.08 ng/g) had higher glycine than the infested while the infested *Irvingia* (11.07 ng/g) had higher value than the uninfested. The Aspartic acid content in *Citrus* revealed the infested leaf had significantly higher quantity (α = 0.05) than the uninfested and the same it was for the *Irvingia* leaf. Proline content of the infested *Citrus* leaf was significantly higher than the uninfested while it was similar in the infested and uninfested *Irvingia* leaves. Cysteine in the infested *Citrus* (9.16 ng/g) was significantly higher than the uninfested whereas the infested and uninfested *Irvingia* possessed cysteine content that was not significantly different. Isoleucine (7.97 ng/g) in the infested *Citrus* was significantly higher than the uninfested but *Irvingia* had similar isoleucine content in both the infested and uninfested state. Pinitol was significantly higher in the infested leaves of the *Citrus* and *Irvingia* hosts.

TABLE III: Free Amino acids and sugar alcohol in the infested and uninfested *Citrus sinensis* and *Irvingia gabonensis* hosts

Sample	Tyrosine ng/g	Phenylalanine ng/g	Serine ng/g	Glycine ng/g	Aspartic Acid ng/g	Proline ng/g	Cysteine ng/g	Isoleucine ng/g	Pinitol ng/g
Infested Leaf of *Citrus sinensis*	12.26^{b}	12.81^{c}	11.49^{b}	9.34^{c}	12.21^{a}	8.91^{a}	9.16^{b}	7.97^{c}	6.78^{b}
Uninfested Leaf of *Citrus sinensis*	10.76^{c}	14.15^{b}	10.55^{c}	10.08^{b}	9.09^{c}	6.88^{c}	8.27^{c}	6.24^{d}	5.76^{c}
Infested Leaf of *Irvingia gabonensis*	14.70^{a}	16.97^{a}	13.49^{a}	11.07^{a}	11.23^{b}	7.89^{b}	10.83^{a}	8.83^{a}	7.98^{a}
Uninfested Leaf of *Irvingia gabonensis*	12.93^{b}	12.71^{c}	11.95^{b}	10.31^{b}	9.79^{c}	7.42^{bc}	10.34^{a}	8.27^{b}	6.87^{b}

Means followed by the same letter in each column are not significantly different by DMRT at α = 0.05

SDS- PAGE electrophoretic bands in Plate 1 ranged from 14.7 – 150 kDa molecular weights. Sample 1 lacked protein at the 20.0 kDa band. Samples 2 and 3 lacked protein at the 20.0 and 25.0 kDa bands. Samples 4 – 6 displayed similar features as they lacked protein at 20.0 kDa band. The protein bands in the samples 1 - 3 (infested *Citrus*) were less densely expressed than those in samples 4 - 6 (uninfested *Citrus*). Generally the leaf specimens of *Citrus* (samples 1 - 6) lacked protein at the 20.0 kDa band. In addition however, the infested *Citrus* leaf specimens lacked protein at 25.0 kDa band.

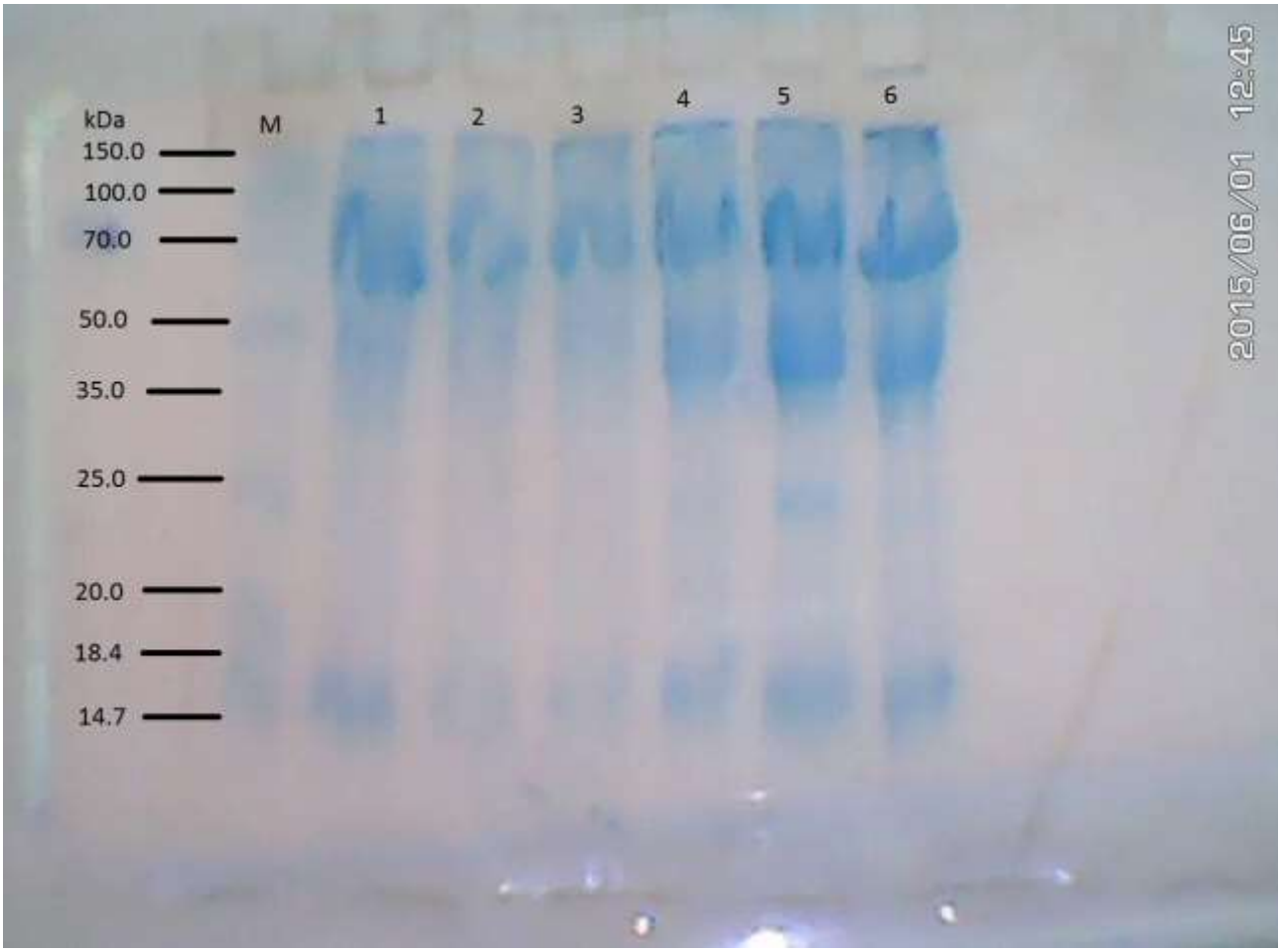

PLATE I: Electrophoregram of the leaf of *Citrus sinensis*
M: Standard; 1, 2 & 3: leaves of infested *Citrus*; 4, 5, & 6: leaves of uninfested *Citrus*

DISCUSSION

Preliminary phytochemical screening of secondary metabolites as well as the quantitative determination of same metabolites from mistletoe and its hosts revealed variations stemming from each host-parasite association. Generally, the mistletoe possessed higher quantities of secondary metabolites when compared with its *Citrus* and *Irvingia* hosts. In a comparative assessment of the constituent metabolites in each mistletoe – host pair vis-a-vis the *Citrus* and *Irvingia* hosts; metabolites such as tannin, phenol, alkaloids, flavonoids and steroids were found to be of significantly higher quantities in the mistletoe on *Citrus* while the other metabolites such as saponin, oxalate and phytate were available in higher quantities in the mistletoe on *Irvingia*. Similarly, distributions of secondary metabolites, within the two host plants were similar. The available metabolites in the mistletoe which are mainly host dependent have been the major reason for strong consideration of host source in the utilization of mistletoe for pharmacognosy and ethnomedicine in places where adopted. Differential accumulation of secondary metabolites in the mistletoe relative to host source is often thought to avail such mistletoe the desired potentials which favour their selection in treatment of certain health challenges (Adodo, 2004; Guimaraes *et al*., 2007). The output of secondary metabolites from *T. bangwensis* on the basis of its association with the *Citrus* and *Irvingia* hosts were consistent with the observation made by some researchers (Wahab *et al*., 2010; Ilesanmi and Olawoye 2011; Umoh *et al*., 2011; Ibrahim *et al*., 2014) who noted that variations existed in the content of secondary metabolites from same mistletoe species occurring on different host plants. Again this observation supports the suggestion of the translocation of pharmacologically active substances from host plants to parasites via their vascular connecting systems at structures such as the haustorium (Bussing and Schietzel, 1999).

Evaluation of the free amino acids and stress metabolite contents of the host-parasite plants showed that the presence and interaction of mistletoe with the host plants do exert some measure of influence on the cumulative content of metabolites in the host plants. Hence, the infested host plants of *Citrus and Irvingia* which bore the presence of mistletoe accrued significantly higher quantities of amino acids and stress metabolites when compared with the uninfested of the same host plants.

Although for phenylalanine and glycine in *Citrus* the results were in contrast. The free amino acids and stress metabolite released were distinctly higher for the infested hosts in the *Citrus and Irvingia* host-parasite association. This result is in support of similar investigations by researchers such as Griffin *et al*., (2004), Liu *et al*., (2008), Usadel *et al*., (2008), Lugan *et al*., (2010) and Murugan *et al*., (2014) which revealed increased accumulation of free amino acids, soluble sugar and sugar alcohols in plants as metabolic responses or otherwise physiological acclimation to various environmental stresses (both abiotic and biotic). The higher free amino acids content in the infested samples is a cue that the activities of the mistletoe might have caused denaturation or breakdown of proteins and bound amino acids, resulting in enhanced free amino acids content of the host tissues.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for soluble proteins carried out on the leaves of infested and uninfested *Citrus* showed products of distinct protein profile expressions relative to the different class of the host plant. The protein banding pattern of the infested host differed sharply from the uninfested. The protein bands of the infested were less densely expressed. The uninfested only lacked Protein at 20.0KDa while protein of 20.0KDa and 25.0KDa were lacking in the infested. This result thus supported the claim of variations in number and intensity of SDS electrophoretic bands of proteins observed between infected and healthy plants (Sharaf *et al*., 2009; Murugan *et al*., 2014). The mistletoe's presence and activities which could be said to induce perturbation, metabolic reaction and proteolytic realignment in the sampled plants is by extension seen to have physico-chemical implication for the host-parasite association. However, no protein bands were noticed in the outcome of the SDS-PAGE process on the leaves of *Irvingia gabonensis*. This does not preclude the existence of soluble proteins in the sample but such might result from the interplay of some intrinsic factors such as high water content, absence of reducing agents (dithiothreitol or β-mercaptoethanol buffer) that breaks disulfide bonds, protein structure, post transitional modification and amino acid composition which minimizes the effect of secondary structure on migration (Osawaru *et al*., 2012).

CONCLUSION

Secondary metabolites observed in the African mistletoe, *Tapinanthus bangwensis* and its hosts were similar; this gives credence to the suggestion of movement of pharmacologically active compounds in the host-parasite association and as such it is therefore logical to state that there are intercellular exchanges of macromolecular substances between mistletoe and its hosts. Increased accumulation of free amino acids as the observed incidence in the infested hosts in comparison with the uninfested implicates the activities of mistletoe in the breakdown of bound amino acids or proteins in the notable variation of protein bands between the infested and uninfested. This observed change in the protein and amino acids status of the infested hosts is a part of plant response to stress which therefore implies that the presence of *Tapinanthus bangwensis* on host plants imposes biotic stress on such host.

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