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Full Length Research Paper

The effectiveness of polyethylene glycol (PEG) and polyvinyl polypyrrolidone (PVPP) on removal of tannins from leaf extracts of selected medicinal plants in Limpopo Province

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Five selected plant species (*Balanites maughamii*, *Breonadia salicina*, *Dombeya rotundifolia*, *Hyperacanthus amoenus* and *Piliostigma thonningii*) were extracted using 70 and 100% acetone and the presence of tannins were determined using radial diffusion assay. Our comparative assessment indicated that plant extracted with 100% acetone showed the highest levels of tannins as compared to 70% acetone. The highest tannin levels in both 70 and 100% acetone extracts were recorded for *P. thonningii*, while no tannins were detected in *B. maughamii*. However, addition of polyethylene glycol (PEG) and polyvinyl polypyrrolidone (PVPP) to the extraction media resulted in the reduction of tannins in all crude plant extracts, with PEG being more effective than PVPP. 1% PEG removed the highest quantity of tannins in all plant extracts, in comparison with 0.5% PEG (63.3%) and 0.5 and 1% PVPP (24.8 and 49.4%, respectively).

Key words: Acetone, tannin, polyethylene glycol (PEG), polyvinyl polypyrrolidone (PVPP).

INTRODUCTION

Tannins are complex group of plant secondary metabolites, which are soluble in polar solutions and these are distinguished from other polyphenolic com-pounds by their ability to precipitate proteins (Silanikove et al., 2001). Tannins in plants occur widely in vascular plants, their occurrence in the angiosperms is particularly associated with woody tissues. They are found in approximately 80% of woody and 15% of herbaceous dicotyledonous species and can occur at high levels in some forages, feeds, foods and medicinal herbs (Bryant et al., 1992; Chung et al., 1998a). Examples of families of dicotyledons rich in tannins are: Leguminosae [*Acacia* species (wattle)], *Sesbania* species, *Lotus* species (trefoil), *Onobrychis* species (sainfoin), Anacardiaceae [(*Scinopsis balansae* (quebracho)], Combretaceae (myrobalan), Rhizophoraceae (mangrove) and Myrtaceae (*Eucalyptus* species, *Mirtus* species (Myrtle) and Polinaceae (canaigre). The amount and type of tannins synthesized by plants varies considerably depending on plant species, cultivars, tissues, stage of development and environmental conditions (Cornell, 2000).

Plant parts containing tannins include bark, wood, fruit, fruit pods, leaves, roots and plant galls (Romani et al., 2012). Tannins are present in the upper epidermis of the leaves. However, in evergreen plants, these secondary metabolites are evenly distributed in all leaf tissues. In the plant cell, tannins are located in the vacuoles, which keep them separately from the proteins and enzymes of the cytoplasm.

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The protein precipitation capacity of tannins has been suggested as an important factor in reducing the palatability of plants by herbivores (Robbins et al., 1987) and in protection of plants against predators (Muetzel and Becker, 2006). This capacity may vary depending on the chemical structure of the compound. When plant tissue is damaged during animal feeding, the tannin may react with the protein moiety of the cell enzymes (oxido-reductases) in the cytoplasm and in the cell wall, making the protein less accessible to the digestive juices of the animal. By binding to cell walls, tannins also reduce the digestion of energy-rich products of microbial fermentation such as volatile fatty acids. This in turn may adversely affect the preference of the feed containing the tannins (Kumar and Vaithiyanathan, 1990; Reed, 1995). Tannins may also bind to bacterial adhesions, and so interfere with the availability of receptors on the cell surface (Cowan, 1999). Tannins at low concentrations may also reduce bacteriophages (bacterial viruses) which can cause a reduction in microbial efficiency through non-specific lysis of bacteria or have anti-protozoal activity (Makkar et al., 1995).

Plants containing more than 10% tannins may have potential adverse effects on humans including stomach upset, renal damage, hepatic necrosis and an increased risk of esophageal and nasal cancer (Kemper, 1999). Many human physiological activities, such as stimulation of phagocytic cells, host-mediator tumor activity and a wide range of anti-infective actions, have been attributed to tannins (Haslam, 1996). In medicine, the tannin-containing plant extracts are used as astringents and diuretics (for example *Crataegus* spp. and *Filipendula ulmaria*), anti-inflammatorys (*Camellia sinensis*), antiseptic (*Camellia sinensis*), and haemostatic pharmaceuticals (*Polygonum aviculare*) against diarrhoea and nasopharyngeal tumors (*Acacia farnesiana*) (Haslam, 1989; Saijo et al., 1989; Hatano et al., 1991; Okuda et al., 1991).

Chemically, there are two main types of tannins: condensed tannins (proanthocyanidins) and hydrolysable (gallotannins and ellagitannins) tannins. Condensed and hydrolysable tannins may occur in the same plant. Proanthocyanidins are more widely distributed than hydrolysable tannins.

In this paper, we investigated the effectiveness of precipitating agents, polyethylene glycol (PEG) and polyvinyl polypyrrolidone (PVPP), on removal of tannins from different crude plant extracts.

MATERIALS AND METHODS

Plant collection

Plant leaves were collected from their natural populations located in the same geographical locations of the Basani village in the District Council of Mopani, Limpopo Province during the summer on a sunny day after all traces of moisture has evaporated. Collected plants were identified using literature and specimen in the herbarium at University of Limpopo (Turfloop Campus). Voucher specimen of the plants were prepared and deposited at the University herbarium.

Fresh collected plant material was examined and the old, insect and fungus-infected leaves were removed. Leaves were dried at room temperature (25°C) for about a week in a forced air draught in a purpose-built drying machine until the leaves were brittle enough to break easily. The dry plant materials were ground to pass through a sieve of 1 mm using laboratory grinding mill (SK 100 standard GuBeisen) and stored in airtight bottles until further extraction.

Extraction procedure

Separate aliquots of finely ground plant material (2 g) were extracted with 50 ml of aqueous 70 or 100% acetone into a conical flask. All conical flasks were sonicated in an ultrasonic bath (Bransonic 220) at room temperature for 30 min followed by shaking of the extract on a Labcon platform horizontal shaker for 1 h. Extracts were then centrifuged at 1600 × g for 15 min. The supernatants were collected and placed in round bottom flasks and evaporated to dryness under reduced pressure on a rotavapor (Optolabor) at 40°C. Dry yields were dissolved in 70 and 100% acetone, respectively, to a final concentration of 50 mg ml^{-1}. These crude extracts were used for determination of tannins.

Addition of PEG and PVPP to plant extracts for removal of tannins

Two tannin-binding chemical agents, PEG and PVPP, were used *in vitro* to test their effectiveness in the removal of tannins from the crude plant extracts in order to select the chemical agent and suitable concentration with the highest tannin binding capacity. Polyethylene glycol (PEG) and PVPP (Sigma) were either added to the plant material, from the beginning of the extraction procedure (variant 1) or to the final crude extract (variant 2) to make final concentrations of 0.5 and 1%. In the later one, the crude extracts with and without PEG and PVPP, were incubated on Thermo-mixture at 37°C for an hour. Extracts without PEG or PVPP were used as controls for each plant sample.

Determination of tannins

Tannins were determined by radial diffusion assay according to Hagerman (1987).

Preparation of agarose plates

Agarose plates were prepared using 1% agarose solution containing 0.1% bovine serum albumin. Agarose was dissolved in acetate buffer pH 5.0 containing 0.05 M acetic acid and 60 µM ascorbic acid. Agarose solution was heated with continuous stirring until the agarose dissolved (melting point at 86°C) and the solution became clear. The solution was then cooled to 45°C in water bath. Bovine serum albumin (0.5 w/v) from Sigma was then added to the heated solution and stirred gently until it dissolved completely. To prevent solidification of the agarose, temperature of the solution was kept at 35°C. The agarose solution (30 ml) was dispensed into sterile Petri dishes (90 mm) on a flat surface. Care was taken to prevent formation of bubble while transferring the agarose solution containing bovine serum albumin. The plates were allowed to cool for 30 min at room temperature and then closed and sealed with parafilm, the plates could now be stored for two to three weeks in refrigerator without losing sensitivity of the assay.

Table 1. Tannins in 70% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1).

Plant species	PEG and PVPP (%)	Tannin level (cm^2)			
		PEG		PVPP	
		Tannin level (cm^2)	Percentage of tannin removal	Tannin level (cm^2)	Percentage of tannin removal
B. m	0.0	0.00^{f}	0.00	0.00^{h}	0.00
	0.5	0.00^{f}	0.00	0.00^{h}	0.00
	1.0	0.00^{f}	0.00	0.00^{h}	0.00
B. s	0.0	2.12^{b}	0.00	2.12^{c}	0.00
	0.5	1.54^{c}	27.4	1.81^{d}	15.0
	1.0	1.06^{e}	50.0	1.56^{e}	26.4
D. r	0.0	2.06^{b}	0.00	2.06^{c}	0.00
	0.5	1.56^{c}	24.3	1.79^{d}	13.1
	1.0	1.20^{d}	41.7	1.59^{e}	22.8
H. a	0.0	2.07^{b}	0.00	2.07^{c}	0.00
	0.5	1.58^{c}	23.7	1.29^{f}	*37.7*
	1.0	1.09^{e}	47.3	1.17^{g}	43.5
P. t	0.0	3.38^{a}	0.00	3.38^{a}	0.00
	0.5	1.24^{d}	63.3	2.54^{b}	24.8
	1.0	1.07^{e}	68.3	1.71^{d}	49.4

B. m = Balanites maughamii; B. s = Breonadia salicina; D. r = Dombeya rotundifolia; *H. a = Hyperacanthus amoenus*; *P. t = Piliostigma thonningii.* Column denoted by the same superscripts are not significantly different ($P < 0.05$).

Radial diffusion assay

Four uniform wells per plate (6.8 mm in diameter) were made on solidified agarose using cork borer. 75 µl of each plants extracts was placed into the wells using micropipette. The selection of this volume was based on preliminary tests where different volumes of plant extracts were used. Petri dishes were covered and sealed with parafilm and were incubated on a horizontal platform in an incubator at 30°C for 96 h until the reaction was stable. The presence of tannins was indicated by the formation of an opaque zone around the wells. Diameter of this area was measured using veneer caliper. The level of the tannins in each samples were expressed in terms of an average diameter square (cm^2) (Hagerman, 1987). 16 replicates were made for each sample.

RESULTS AND DISCUSSION

Table 1 shows the tannin levels in 70% acetone extracts from five plants in the presence and absence (controls) of tannin binding reagents (PEG and PVPP) at concentration of 0.5 and 1.0% (w/v) (variant 1). Extract of *Piliostigma thonningii* showed the highest levels of tannins (3.38 cm^2) among all plant extracts, while no tannins were recorded in 70% acetone extract of *B. maughamii*. There were no significant differences ($P < 0.05$) in tannin levels between *B. salicina*, *D. rotundifolia* and *H. amoenus* (Table 1).

Effect of PEG and PVPP on binding tannins from 70% acetone extract when added at the beginning of the extraction (variant 1)

Addition of PEG and PVPP to the extraction media (variant 1) resulted in reduction of tannins in all crude plant extracts with PEG being more effective than PVPP. The percentage of tannin removal depended on the type and the concentration of the precipitating agent used. In general, 1% PEG appeared to remove higher percentages of tannins in all plant extracts, in comparison to 0.5% PEG and 0.5 and 1% PVPP (24.8 and 49.4), respectively. The highest percentage of tannin removal (68.4) was achieved with 1% PEG in *P. thonningii*. 1% PVPP resulted in lower percentage (49.4) of tannin removal for this extract. Similar results were observed for *B. salicina* and *D. rotundifolia* where addition of 1% PVPP resulted in lower percentage (15.0 and 13.1) of tannin removal. In particular, 0.5% PVPP followed by 0.5% PEG were less effective on tannin removal from all the 70% acetone plant extracts than 1% PVPP and 1% PEG.

Crude plant extracts prepared with 100% acetone showed similar pattern of variation of tannins between plant samples as compared to 70% acetone extracts except for *B. maughamii* which showed no presence of tannins (Table 2). However, higher tannins levels were

Table 2. Tannins in 100% acetone extracts from selected plant samples in the presence and absence of PEG and PVPP added from the beginning of the extraction (variant 1).

Plant species	PEG and PVPP (%)	Tannin level (cm^2)			
		PEG		PVPP	
		Tannin level (cm^2)	Percentage of tannins removal	Tannin level (cm^2)	Percentage of tannins removal
B. m	0.0	0.00^{j}	0.00	0.00^{j}	0.00
	0.5	0.00^{j}	0.00	0.00^{j}	0.00
	1.0	0.00^{j}	0.00	0.00^{j}	0.00
B. s	0.0	3.43^{b}	0.00	3.43^{b}	0.00
	0.5	1.06^{f}	69.1	2.17^{e}	63.3
	1.0	0.94^{g}	72.6	1.87^{f}	54.5
D. r	0.0	2.41^{d}	0.00	2.41^{d}	0.00
	0.5	0.93^{gh}	61.4	1.83^{g}	24.1
	1.0	0.71^{i}	70.5	1.04^{i}	56.8
H. a	0.0	2.67^{c}	0.00	2.67^{c}	0.00
	0.5	0.91^{gh}	41.2	1.84^{fg}	31.1
	1.0	1.57^{e}	66.0	1.56^{h}	41.6
P. t	0.0	4.29^{a}	0.00	4.29^{a}	0.00
	0.5	0.90^{h}	79.0	2.42^{d}	43.6
	1.0	0.69^{i}	84.0	1.53^{h}	64.3

B. m = Balanites maughamii; B. s = Breonadia salicina; D. r = Dombeya rotundifolia; *H. a = Hyperacanthus amoenus*; *P. t = Piliostigma thonningii.* Column denoted by the same superscripts are not significantly different at $P < 0.05$.

recorded in all plant samples extracted with 100% acetone than with 70%. In *P. thonningii* and *B. salicina* plant samples, the diameter square of the area of precipitation which reflects the level of tannins was increased from 3.38 to 4.29 and from 2.12 to 3.43, respectively (Table 3). Unlike in 70% acetone extracts, tannin levels in 100% acetone extracts from all plant samples differed significantly (Table 2).

Effect of PEG and PVPP on binding tannins from 100% acetone extracts when added at the beginning of the extraction (variant 1)

Both PEG and PVPP were more effective in the removal of tannins from 100% acetone extracts (up to 84% for *P. thonningii*) than from 70% acetone extracts (up to 64% for *P. thonningii*). However, similarly to the 70% acetone extracts, 1% PEG proved to be more effective than 0.5% PEG, 0.5 and 1% PVPP in reducing tannin levels in the crude plant extract, resulting in the highest percentage of tannin removal up to 84% for *P. thonningii*.

Effect of PEG on binding tannins from 100% acetone extract when added to the final extracts (variant 2)

Tannin levels in plant extracts with or without 1% PEG when added to the final crude plant extract instead of the plant material from the beginning of the extraction procedure, resulted in 100% removal of tannins in all plant samples since no precipitation areas around the wells were recorded.

Conclusion

Comparative assessment of tannins in both 70% aqueous acetone and 100% acetone extracts was done in this study in order to select appropriate extraction procedure suitable for both assays. Our results show higher level of tannins in 100% acetone extracts from all plant samples than in 70% acetone extracts. Based on the results and considering the reports on plant extracts used for antibacterial assays, 100% acetone was selected as an extraction solvent for preparation of all plant since it appeared to be suitable for tannin assay. The highest tannin levels in both 70 and 100% acetone extracts was recorded for *P. thonningii*, while no tannins were detected by radial diffusion assay in *B. maughamii*. This could be due to the presence of insoluble tannins since in many plants, more than 50% of tannins are reported to be not extractable due to insolubility (Cornell, 2000).

Two tannin-binding reagents (PEG and PVPP) at different concentrations (0.5 and 1%) were used to

Table 3. Tannins in 70 and 100% acetone extracts from selected plant samples in the absence of PEG and PVPP (controls).

Solvent (%)	Plant species				
	B. m	***B. s***	***D. r***	***H. a***	***P. t***
70	0.00[a]	2.12[b]	2.06[b]	2.07[b]	3.38[b]
100	0.00[a]	3.43[a]	2.41[a]	2.67[a]	4.29[a]

B. m = Balanites maughamii; B. s = Breonadia salicina; D. r = Dombeya rotundifolia; *H. a = Hyperacanthus amoenus*; *P. t = Piliostigma thonningii.* Column denoted by the same superscripts are not significantly different at $P < 0.05$.

remove tannins from 70 and 100% acetone extracts of *B. maughamii*, *B. salicina*, *D. rotundifolia*, *H. amoenus* and *P. thonningii*. Comparative assessment of the effect of the two selected precipitating agents, PEG and PVPP, at two different concentrations (0.5 and 1%) revealed that 0.5% of both PEG and PVPP, removed less tannins in both 70 and 100% acetone extracts (variant 1) as compared to 1% of PEG and PVPP with PEG being more effective than PVPP. Similar results were obtained by Makkar et al. (1995) who used PEG to bind tannins in leaf material of *Acioa barteri*, *Dichostachys cinerea*, *Guiera senegalensis* and *Piliostigma reticulatum*. Polyethylene glycol is reported to have higher binding affinity to proteins as compared to PVPP (Makkar, 2003). Addition of PEG results in the formation of PEG-tannin complexes which inactivates tannins. Polyethylene glycol is reported to react preferentially with condensed tannins and can prevent the formation of tannin-protein complexes (Jones and Mangan, 1977). The addition of 1% PEG to plant samples, at the beginning of the extraction procedure (variant 1) in this study, resulted in the highest percentage of tannin removal, up to 84%, and therefore, it was selected as a precipitating agent for binding tannins. Higher tannin levels were recorded in all plant samples extracted with 100% acetone than with 70% acetone. PEG and PVPP (both at 0.5%) removed less tannin in 100% acetone extracts as compared to 1% PEG and PVPP with PEG being more effective than PVPP.

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