Purification of nuts of *Semecarpus anacardium* Linn., a herbal drug for arthritis

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Although nuts of *Semecarpus anacardium* Linn. (Anacardiaceae) (SA nuts) are used in the Ayurveda and Siddha systems of medicine for the management of arthritis and other inflammatory diseases, they are always associated with several side effects, if used unpurified. Several traditional methods for purification are available, but they are not free from toxicity and also the scientific basis behind these purification steps is not known. Here, we have hypothesized that the oily part of the nut is toxic and its degree of removal is proportional to its safety margin. To test this hypothesis, we treated the SA nuts with brick powders (traditional method of Ayurveda), silica gel and hexane solvent for various time periods. These defatted nuts were washed and extracted with ethanol and the solvent-free extract was tested on rat peritoneal macrophages in terms of NO production, both in the absence and presence of lipopolysaccharides (LPS). Results suggest that the hexane fraction (oily part) is highly toxic and produces free radicals (FR) in a concentration-dependent manner without affecting the cell viability and the hexane fraction works as a prooxidant, similar to that of LPS. Contrary to this, the alcoholic fraction of hexane-washed SA nuts (defatted nuts) significantly scavenged FR and its yield was directly proportional to the removal of the oily part. Thus it is suggested that the oily part of the nuts is prooxidant, whereas the alcoholic fraction is antioxidant, and that the use of hexane is better for purification of SA nuts than the traditional methods, as it enhances the therapeutic value.

Keywords: Arthritis, herbal drug, purification, *Semecarpus anacardium*.

NUTS of *Semecarpus anacardium* Linn. (Anacardiaceae) (SA nuts) are used in the Ayurveda and Siddha systems of medicine, with various therapeutic properties such as anti-helminthic, anti-fungal, anti-carcinogenic, nervous debilities, arthritis and cardiovascular diseases[1–4]. We have previously reported that alcoholic extract of the purified nuts of SA arrests cell cycle in DU 145 prostate cancer cells[5] and one of its products 'Sandhika' has been shown to enhance Ca nodule formation in MC3T3-E1 osteoblast-like cells, both in the presence and absence of lipopolysaccharides (LPS), highlighting its potential to treat osteoporosis[6]. It has also been reported to enhance the reduced phagocytic function of neutrophils in adjuvant arthritis[7,8], lower lipid peroxidation (LPO)[9,10], normalize the enhanced activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and reduce glutathione (GSH) content in arthritic rats[11], suppress LPS-induced nuclear translocation of transcription factors (NFkB)[12] and release nitric oxide (NO) in the culture medium[13]. Further, the role of the oily part with several therapeutic applications has also been shown in Ayurvedic texts[2] and in the recent literature[11]. Though it has several therapeutic applications, its signalling pathway is still unexplored[14]. It is always used with caution, because of the tedious purification process, which is still primitive and has no objective parameter to ascertain its complete purification.

Here, we hypothesize that the oily portion of SA nuts is toxic and its removal improves its therapeutic quality and enhances the safety margin. In the traditional method of purification, as described in the Ayurvedic texts, nuts (only those which do not swim in water) are crushed and mixed with brick powder and kept for a day. The next day they are washed with water and boiled in freshwater with 2–3 changes. Finally, washed nuts are dried and considered as purified nuts. They are powdered and used in the preparation of Ayurvedic formulations[2].

However, to test our proposed hypothesis, we used two approaches. One is a physical method and the other a chemical method of purification. In the former case, we used silica gel in place of brick powder, which is considered to be a better adsorbent of oil. In the latter case, we washed the crushed nuts with hexane for various time intervals. Further, all the nuts (treated using different methods, as described above) were finally extracted with ethanol to determine the yield percentage. The biological potential was also determined in terms of free radical (FR) scavenging potential on LPS-induced NO release in attached rat peritoneal macrophages and inter-group comparison was made.

LPS was purchased from Sigma (USA). Methelyne blue, RPMI-1640 and foetal calf serum (FCS) were purchased from Hi Media (Mumbai, India). Other chemicals were of analytical grade and purchased from Merck (India).

Authentic sample of SA nuts was purchased from the Ayurvedic Pharmacy at the Institute of Medical Sciences, Banaras Hindu University, Varanasi. They were washed and only good quality nuts, which did not float in water, were selected for further study. They were dried, weighed and subjected to various methods of extraction as described in Table 1. One sample was first exhaustively extracted with hexane in a soxhlet extractor and then successively extracted with ethanol. This fraction was considered as the basic extract for comparison of other samples.

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Table 1. Effect of various methods of purification of nuts of *Semecarpus anacardium* on percentage yield of alcoholic extract and its percentage inhibition of lipopolysaccharide-induced nitric oxide production by rat peritoneal macrophages

Method of purification (in each group 2 g of nuts was subjected to different methods of purification and then finally extracted using 10 ml alcohol)	Yield (%)	Inhibition of nitric oxide production (%)
Seed extracted directly using 10 ml alcohol (A)	12.5 ± 3.46	8.06 ± 1.26
Treated with brick powder for one day (B), boiled with water	18.9 ± 3.43	10.61 ± 2.22
Treated with silica gel for one day (C), boiled with water	24.8 ± 3.40	20.17 ± 2.42**
Extracted with hexane for		
Two hours (D)	46.21 ± 3.46	35.90 ± 3.12**
One day (E)	61.2 ± 3.00	51.08 ± 3.24*
Five days (F)	73.8 ± 3.40	64.89 ± 3.02*
Exhaustively extracted with hexane (G)	76.92 ± 3.44	60.99 ± 3.10*

Level of significance ($P^* < 0.01$, $P^{**} < 0.001$) when compared with traditional Ayurvedic methods (B).

Table 2. *Rf* values of different spots of hexane and alcoholic fractions of *S. anacardium* nuts in various solvent systems

Fraction	Solvent system	No. of spots	*Rf* value
Hexane fraction	Hexane + ethyl acetate (6 : 4)	5	0.74, 0.62, 0.43, 0.37, 0.33,
	Hexane + benzene (2 : 8)	3	0.56, 0.44
Alcoholic fraction	Benzene + ethyl acetate (4 : 6)	5	0.81, 0.71, 0.60, 0.38, 0.32,
	Benzene + ethyl acetate (8 : 2)	2	0.32, 0.36

In the case of purification with traditional method, the crushed nuts were mixed with brick powder and kept for 24 h. The next day, they were washed and boiled with distilled water for 2–3 successive changes, as described earlier[13]. Finally, the washed nuts were dried and extracted with a fixed volume of ethanol (10 ml) for 24 h.

For purification using newer methods, a physical method was adopted where the brick powder was replaced with dry silica gel. Other steps were the same as in the previous case described with brick powder. In another set of chemical-purification method, hexane was used to remove the oil.

Here, the crushed nuts were extracted with hexane with periodical shaking for different time periods (1, 2 and 24 h) at room temperature. In another set, they were extracted for 5 days with five sequential changes of hexane solvent. The idea was to have complete extraction at room temperature. Finally these defatted nuts were taken out of the hexane solvent, air-dried and extracted with ethanol for 24 h at room temperature.

This alcoholic extract from each sample was dried to the solvent-free condition and weighed to determine the yield percentage. The extract was further standardized on thin layer chromatography (TLC) and the fingerprint of both extracts was reported as *Rf* value of various spots (Table 2). The TLC fingerprinting was carried out in two solvent systems with the objective to assess the number of spots in the lower and higher polarity conditions. (These spots do not represent the same compound, because while raising the polarity of the developing solvent, spots that developed in lower polarity got merged in the solvent front and a new compound appeared on the plate, which did not move in the low polar solvents and remained confined to the origin of the loading spot.)

Animal experiments were approved by the Institute Ethical Committee for Animal Welfare. Inbred albino rats of CF strain were used to isolate peritoneal macrophages. Next 5 ml sterile ice-cold PBS, devoid of calcium and magnesium ions, was injected to the peritoneal cavity of these rats and their abdomen was squeezed for 5 min. Finally, peritoneal fluid was aspirated out, cells were spin down and re-suspended in complete RPMI 1640 culture medium (supplemented with 5% FCS) and counted. Then 1×10^5 macrophages were dispensed in each cavity of the 96-well flat-bottom culture plates and allowed to stand for 2 h in CO_2 incubator to attach the macrophages. The floating cells were removed and attached macrophages were supplemented with fresh complete culture media and subjected to various treatments as described earlier[13].

All the tests were repeated three times in triplicate. Sham control wells were treated with the same concentration of DMSO (drug vector), whereas experimental wells were pretreated with different extracts (500 μg/ml final concentration) for 30 min and then LPS was added (20 ng/ml). After 24 h, NO content was assessed in terms of nitrite concentration with Griess reagent by taking absorbance at 540 nm on ELISA plate reader[15]. All the data were equalized by viable cell number, which was assessed using methylene blue assay, measured as absorbance at 600 nm on ELISA plate reader (Multiscan, Labsystem).

All data were expressed as means ± SD. Statistical comparison with control was done using SPSS 7.5 for Windows, SPSS Inc.

Table 3. Effect of hexane and alcoholic fractions of SA nuts on cell viability and free radical generation in rat peritoneal macrophages

Treatment group	Cell viability (OD^{550} methylene blue uptake) (mean ± SD)	Free radical generation NO production (NO_2 μM/million cells) (mean ± SD)
Normal cells	1.392 ± 0.986	8.09 ± 1.987
Sham control DMSO	1.402 ± 1.002	10.76 ± 1.587
SA–hexane (ng/ml)		
50	1.389 ± 0.964	10.54 ± 1.539
200	1.390 ± 0.894	20.76 ± 1.543
400	1.386 ± 0.942	42.89 ± 1.943
600	1.392 ± 0.954	43.87 ± 1.749
1000	1.091 ± 1.002	44.04 ± 1.698
SA–alcoholic fraction (μg/ml)		
100	1.398 ± 1.112	10.09 ± 1.975
200	1.401 ± 0.926	19.56 ± 1.968
400	1.387 ± 0.958	15.86 ± 1.058
500	1.266 ± 0.992	16.95 ± 1.867
700	1.020 ± 0.984	16.89 ± 1.592
Only LPS (20 ng/ml)	1.3591 ± 0.886	43.08 ± 1.298
LPS + SA–hexane (600 ng/ml)	1.3261 ± 0.924	40.92 ± 1.42
LPS + SA–alcoholic fraction (500 μg/ml)	1.3661 ± 0.946	18.22 ± 1.624[a]

$P^* < 0.01$, Level of significance when compared with Sham control.
$P^a < 0.01$, Level of significance when compared with LPS.

Let us consider the effect of various treatments of purification on yield percentage of alcoholic extract. Raw nuts that were not subjected to any treatment (Table 1, A) showed yield percentage of alcoholic extract in the range of 12.5% compared to nuts, that were washed for 5 days (Table 1, F), with five successive changes with hexane (73.8%). This was similar to the data obtained from nuts that were exhaustively extracted with hexane in a soxhlet extractor (Table 1, G). Purification using classical Ayurvedic method (treatment with brick powder) showed yield of 19%, which significantly improved when treated with silica gel (24%). However, when these nuts were washed with hexane, they showed much better yield compared to all physical methods, i.e. use of brick powder or silica gel. Here the yield percentage of alcoholic extract was found to gradually increase along with the time of hexane treatment. Even with 1 h hexane treatment it was 43%, which was almost double the value of silica-gel treatment (Table 1, D–F). This suggests that gradual decrease in oil content of nuts enhances the yield percentage of the alcoholic fraction containing polar compounds. This gradual increase in yield percentage of alcoholic fraction with gradual increase in hexane washing may be correlated with the proportional removal in the oil content of the nuts.

In fact, oil content reduces the polarity of added alcohol (fixed volume 10 ml in each tube). Therefore, presence of more oil will finally render lesser polarity of extracting solvent (oil : alcohol = varying volume of oil : 10 ml alcohol). Thus, if the oil content is lesser in the nuts, then there will be more net polarity of extracting solvent, which will result in more extraction of the polar fraction of the nut. However, significantly lesser yield in case of physically-treated nuts (either with brick powder or silica gel) may be because of loss of polar contents in the washing step with hot water. Thus it could be suggested that the traditional method of purification was basically aimed to reduce the oily content of the nuts and it can be replaced by newer techniques of using other adsorbents such as silica gel or organic extraction by non-polar solvents such as hexane.

Now let us consider the effect of various treatments of purification on biological activity of SA nuts. The biological activity of SA nuts was tested against LPS-induced NO production in rat peritoneal macrophages. It showed (Table 1) minimum activity in the extract from unpurified nut (8.06%), which gradually enhanced when treated with brick (10.61) or silica gel (20.17). Interestingly, in the chemically purified nuts (by hexane washing) inhibition in NO production was gradually enhanced with increase in time of hexane washing and activity was maximum when the nuts were washed for 5 days (daily one change with fresh hexane; 64.89%). This was slightly higher than exhaustively extracted nuts (60.99%), which may be due to loss in biological activity of the active ingredient due to hot extraction process compared to room temperature extraction in five-days treatment.

The proportional increase in biological activity along with yield percentage of the alcoholic extract suggests that a biologically active compound may be present in polar fraction of SA nuts. This is evident from an earlier report[16], where it has been shown that tetrahydroamentoflavone[17] and other polar compounds constitute the active principle for their anti-inflammatory property[18,19].

Thus any method which is aimed at enhancing the higher component of the polar fraction, may be beneficial for enhancing biological activity of purified SA nuts. Since here we have tested it on LPS-induced NO production, which is directly involved in the process of inflammation, it could be suggested that the reported anti-inflammatory property of SA nuts[20,21] is associated with

its polar fraction and improved method of purification will lead to better efficacy of the Ayurvedic formulation.

Now consider the effect of different fractions of SA nuts on cell viability and FR generation. The above data suggest that the polar fraction contains biologically active components, but it is not clear whether the non-polar fraction has some antidote compound with opposite effect also or only possesses inactive components. In order to address this question, we further tested both the fractions (non-polar hexane fraction and polar alcoholic fraction) separately on attached macrophages, in presence and absence of LPS.

It was observed that the hexane fraction induces NO production in a concentration-dependent manner, whereas alcoholic extract did not show any such stimulatory effect. However, when tested on LPS-induced NO production, the hexane fraction neither showed any inhibitory nor any additive response, but the alcoholic fraction significantly inhibited NO production (Table 3). This again suggests that the oily portion of SA nuts might have some phyto-molecule, associated with biological activity opposite to that of the polar fraction. Thus our proposed hypothesis holds true that the oily fraction of SA possesses opposite action to that of the polar fraction and the purification step helps to reduce the oily part, which finally enhances the yield of the therapeutically active polar part of the SA nuts.

Therefore, we conclude that the traditional method of purification of SA nuts was mainly focused on reducing its oily part, but it was not efficient. Whereas boiling with water resulted in significant loss of the active constituent and also left the residual part of the oily content intact, which is associated with the reported adverse side effects.

Further, the traditional method using brick powder could be replaced by better oil adsorbents such as silica gel, if manufacturers of Ayurvedic drugs are not allowed to use organic solvents. However, if this is allowed by the *Indian Pharmacopoeia* for making Ayurvedic drugs, then exhaustive washing of SA nuts with hexane or other organic non-polar solvents would lead to significant removal of the oily content of SA nuts, which is associated with the pro-inflammatory property.

These procedures of purification may be adopted by Ayurvedic pharmaceutical companies for making better Ayurvedic formulations with fewer side effects and better therapeutic value.

1. Chopra, R. N., Nayar, S. L. and Chopra, I. C., *Glossary of Indian Medicinal Plants*, CISR, New Delhi, 1956, p. 225.
2. Pandey, G. S. and Chunekar, K. C. (eds), *Bhav Prakash Nighantu*, Chaukambha Vidya Bhavan, Varanasi, 1967, pp. 139–141.
3. Arulkumaran, S., Ramprasath, V. R., Shanthi, P. and Sachdanandam, P., Alteration of DMBA-induced oxidative stress by additive action of a modified indigenous preparation – Kalpaamruthaa. *Chem. Biol. Interact.*, 2007, **25**, 99–106.
4. Sowmyalakshmi, S., Nur-E-Alam, M., Akbarsha, M. A., Thirugnanam, S., Rohr, J. and Chendil, D., Investigation on *Semecarpus* lehyam – A Siddha medicine for breast cancer. *Planta*, 2005, **220**, 910–918.
5. Tripathi, Y. B., Tripathi, P., Reddy, M. V. R., Tewari, D. S. and Reddy, E. P. K., Effect of *Semecarpus anacardium* on cell cycle of DU-145 cells. *Phytomedicine*, 1998, **5**, 383–388.
6. Tripathi, Y. B., Tripathi, P., Korlagunta, K., Chai, S. C., Smith, B. J. and Arjmandi, B. H., Role of Sandhika: A polyherbal formulation on MC3T3-E1 osteoblast-like cells. *Inflammation*, 9 August 2007 [Epub ahead of print].
7. Ramprasath, V. R., Shanthi, P. and Sachdanandam, P., Effect of *Semecarpus anacardium* Linn. nut milk extract on rat neutrophil functions in adjuvant arthritis. *Cell Biochem. Funct.*, 2006, **24**, 333–340.
8. Selvam, C., Jachak, S. M. and Bhutani, K. K., Cyclooxygenase inhibitory flavonoids from the stem bark of *Semecarpus anacardium* Linn. *Phytother. Res.*, 2004, **18**, 582–584.
9. Tripathi, Y. B. and Singh, A. V., Effect of *Semecarpus anacardium* nuts on lipid peroxidation. *Indian J. Exp. Biol.*, 2001, **39**, 798–801.
10. Tripathi, Y. B., Reddy, M. M., Pandey, R. S., Subhashini, J., Tiwari, O. P., Singh, B. K. and Reddanna, P., Anti-inflammatory properties of BHUx, a polyherbal formulation to prevent atherosclerosis. *Inflammopharmacology*, 2004, **12**, 131–152.
11. Ramprasath, V. R., Shanthi, P. and Sachdanandam, P., *Semecarpus anacardium* Linn. nut milk extract, an indigenous drug preparation, modulates reactive oxygen/nitrogen species levels and antioxidative system in adjuvant arthritic rats. *Mol. Cell Biochem.*, 2005, **276**, 97–104.
12. Singh, D., Aggarwal, A., Mathias, A. and Naik, S., Immunomodulatory activity of *Semecarpus anacardium* extract in mononuclear cells of normal individuals and rheumatoid arthritis patients. *J. Ethnopharmacol.*, 2006, **108**, 398–406.
13. Tripathi, Y. B. and Pandey, R. S., *Semecarpus anacardium* L. nuts inhibit lipopolysaccharide-induced NO production in rat macrophages along with its hypolipidemic property. *Indian J. Exp. Biol.*, 2004, **42**, 432–436.
14. Smit, H. F., Woerdenbag, H. J., Singh, R. H., Meulenbeld, G. J., Labadie, R. P. and Zwaving, J. H., Ayurvedic herbal drugs with possible cytostatic activity. *J. Ethnopharmacol.*, 1995, **47**, 75–84.
15. Griess, J. P., Bemerkungen zu der Abhandlung der HH: Wesely und Benedikt 'Über einige Azoverbindungen'. *Ber. Dtsch. Chem. Ges.*, 1879, **12**, 426–428.
16. Shanthi, P., Ramprasanth, V. R. and Sachdanandam, P., Nut milk extract on the changes associated with collagen and glycosaminoglycan metabolism in adjuvant arthritic Wistar rats. *Chem. Biol. Interact.*, 2006, **162**, 43–52.
17. Selvam, C. and Jachak, S. M., A cyclooxygenase (COX) inhibitory biflavonoid from the seeds of *Semecarpus anacardium*. *J. Ethnopharmacol.*, 2004, **95**, 209–212.
18. Vijayalakshmi, T., Muthulakshmi, V. and Sachdanandam, P., Effect of the milk extract of *Semecarpus anacardium* nut on adjuvant arthritis – A dose-dependent study in wistar albino rats. *Gen. Pharmacol. Vasc. Syst.*, 1996, **27**, 1223–1226.
19. Ramprasath, V. R., Shanthi, P. and Sachdanandam, P., Evaluation of antioxidant effect of *Semecarpus anacardium* Linn. nut extract on the components of immune system in adjuvant arthritis. *Vasc. Pharmacol.*, 2005, **42**, 179–186.
20. Ramprasath, V. R., Shanthi, P. and Sachdanandam, P., Therapeutic effects of *Semecarpus anacardium* Linn. *Phytochemistry*, 1995, **39**, 405–407.
21. Selvam, C., Jachak, S. M. and Bhutani, K. K., Cyclooxygenase inhibitory flavonoids from the stem bark of *Semecarpus anacardium* Linn. *Indian J. Physiol. Pharmacol.*, 1983, **27**, 83–91.

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